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Cold defence responses: the role of oxidative stress

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1. ABSTRACT

Low temperatures provoke increased production of heat accompanied by increased respiration, oxygen consumption and the production of partially reduced oxygen species called ROS. ROS induce different forms of cellular oxidative damage, disturb the redox state and can change the activity of several metabolic enzymes. Organisms have developed a functionally connected set of anti-oxidant enzymes and low molecular mass compounds (together termed the ADS) that metabolise primary ROS. If ROS production within cells overwhelms the ADS, oxidative damage arises and oxidative stress can occur. Short-term cold exposure in endotherms leads to oxidative stress. As cold exposure persists organisms develop adaptive changes toward reducing ROS production and increasing the ADS. In contrast, heterotherms and ectotherms as a normal part of their over-wintering strategy slow down metabolism, oxygen consumption and subsequently cause ROS production. Increased baseline activity of key anti-oxidant enzymes as well as 'secondary' enzymatic defence and/or glutathione levels in preparation for a putative oxidative stressful situation arising from tissue re-oxygenation seems to be the preferred evolutionary adaptation of such animals exposed to low environmental temperatures.

2. INTRODUCTION

Stable conditions favour survival of a biological system within its environment. According to non-equilibrium thermodynamics internal homeostasis describes a stationary state of non-equilibrium. For spontaneously stable states to occur a lowered metabolic rate, withdrawal of sympathetic drive and reinforcement of vagal heart tone and circulation are required. This confirms that the parasympathetic division of the autonomic nervous system is the main controller of homeostasis (1). Heat production occurs in living organisms and the "metabolic rate" is commonly defined as "the rate of heat production" (2). The latter depends on the level of adenosine triphosphate (ATP) that is mainly produced by mitochondrial oxidative phosphorylation. Inevitably the production of partially reduced oxygen and the formation of ROS occurs. However, respiration is not just about generating energy, it's also about generating feedback that stimulates a cell to recognise and respond to its environment and blocking respiration generates chemical signals in the form of ROS (3). Precise relationships between heat production, ROS, the ADS and physiological states are still poorly defined. Low temperatures provoke physiological responses related to heat production, induce phase changes in energy production, oxygen consumption and subsequent ROS

production. Exposure to low temperature alters the ADS activity, its composition and its structure with respect to its individual components which strive to provide the best possible protection and preservation of cellular homeostasis. By understanding the mechanisms of physiological regulation, underlining cold defence can provide information regarding the regulation of cellular homeostasis, particularly with respect to hibernation and insect dormancy. A reduction in the relative mass of heat-producing organs in heat-adapted animals has been found to be associated with a reduction in the metabolic rate and a similar reduction in the oxidative enzyme activity in liver mitochondria and liver glucose 6-phosphatase activity. Adaptation to 30°C, from the point of view of mitochondrial protein synthesis, is characterised by the disbalance between its rate of synthesis and degradation and its decreased ATPase activity as a consequence of depressed thyroid activity in warm conditions. In general, chemical thermo-suppression seems to occur in cells of various organs and tissues during heat acclimation (4). On the other hand, metabolic suppression in cold-adapted animals, hibernators or insects, in the form of dormancy is poorly understood. Furthermore, cold survival mechanisms are complex involving anti-oxidant molecules. Future studies examining cold exposure, cold adaptation and animal hibernation will establish detailed relationships between heat production, ROS production, ADS activity and these related processes.

2.1. Reactive oxygen species (ROS)

During metabolism production of superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), lipid radicals ($L^{\cdot}$) and peroxides (LOOH) occurs. Cellular ROS generators are diverse; the principal generator is the mitochondrial respiratory process, which yields $O_2^{\bullet-}$ (5,6). However, mitochondrial ROS production is not a simple bi-product of mitochondrial respiration and oxygen consumption as total rates of ROS generation in different tissues are not linked to their differences in oxygen consumption (7). *In vivo* the one-electron reduction of O_2 to $O_2^{\bullet-}$ is thermodynamically favoured and mitochondrial $O_2^{\bullet-}$ production takes place at redox-active prosthetic groups within proteins or when electron carriers such as reduced coenzyme Q ($CoQH_2$) are bound to proteins (8). The flux of $O_2^{\bullet-}$ within the matrix of mammalian mitochondria is related to the concentration of potential electron donors including the $NADH/NAD^+$ and $CoQH_2/CoQ$ ratios, the local concentration of O_2 and the second-order rate constants for the reactions between them. Significant $O_2^{\bullet-}$ production arises from complex I when the mitochondria do not produce ATP, have a high proton motive force and a reduced CoQ pool and when there is a high $NADH/NAD^+$ ratio in the mitochondrial matrix (9). Therefore, the production of $O_2^{\bullet-}$ in mitochondria is continuous, but its extent depends on the cellular redox state and the amount of available O_2. Many enzymes and substrates are linked to $O_2^{\bullet-}$ production in mitochondria. *In vivo* substrate supply to the respiratory chain is controlled by physiological factors, diet and hormonal status leading to alterations in the steady state reduction potential of mitochondrial electron carriers. As the main role of mitochondria is to synthesise ATP, it has been suggested that this mode of $O_2^{\bullet-}$ production might account for most of the overall exposure of mitochondria to $O_2^{\bullet-}$. The maximum $O_2^{\bullet-}$ production rate *in vivo* is proportional to the content of respiratory complexes such as complex I and thus correlates with the maximum respiration rate. Little is known about the actual flux of $O_2^{\bullet-}$ within mitochondria *in vivo*, about how it changes under different physiological circumstances, or about its quantitative importance relative to other sources of ROS. $NADH/NAD^+$, $CoQH_2/CoQ$ ratios and the local O_2 concentration are all highly variable and difficult to measure *in vivo*. Therefore, the estimation of $O_2^{\bullet-}$ generation in isolated mitochondria *in vivo* from $O_2^{\bullet-}$ production rates is not possible (6).

$O_2^{\bullet-}$ itself is relatively un-reactive but is a great promoter of further cellular oxidative reactions. The cytotoxicity of $O_2^{\bullet-}$ *in vivo* comes from its protonated form $HO_2^{\bullet}$ formed via the Haber-Weiss and Fenton reactions, the production of peroxynitrite ($ONOO^-$) from nitric oxide ($NO^{\bullet}$) and the ability of $O_2^{\bullet-}$ to oxidise $[4Fe\text{-}S]^{2+}$ clusters of several enzymes leading to release of iron which can further promote Fenton chemistry (10). The other two ROS originating from the above-mentioned reactions are H_2O_2 and $OH^{\bullet}$. The former is also generally poorly reactive. It crosses cell membranes by diffusion or facilitated by aquaporins and initiates further cellular oxyradical reactions. In the presence of iron (Fe^{2+}) or copper (Cu^+) H_2O_2 can be split into $OH^{\bullet}$ and hydroxyl anions (OH^-), the latter being the main cellular precursor of $OH^{\bullet}$ (11). Once generated $OH^{\bullet}$ reacts with molecules in their immediate vicinity generating and promoting site-specific oxidative damaged molecules (12). If $OH^{\bullet}$ collides with a membrane lipid (LH) molecule a self-propagated lipid peroxidation chain reaction generates lipid peroxides resulting in the disturbance of membrane function. If not eliminated ROS can induce oxidative damage within every class of all cellular molecules.

ROS are capable of inhibiting or changing the activity of several metabolic enzymes. H_2O_2 inhibits glyceraldehyde 3-phosphate dehydrogenase by oxidation of its -SH groups (13). ROS also affect ADS enzymes that are responsible for modulating the extent and types of ROS in cells. A high level of $O_2^{\bullet-}$ can inactivate catalase (CAT) (14). Cu,Zn-containing superoxide dismutase (CuZnSOD or SOD1) activity decreases due to inactivation by $OH^{\bullet}$ and H_2O_2 (15,16). $NO^{\bullet}$ reversibly binds to the haem groups within CAT and decreasing its activity. In addition, $ONOO^-$ can inhibit CAT. Manganese-containing superoxide dismutase (MnSOD or SOD2) stimulates $NO^{\bullet}$ decay leading to partial enzyme inactivation, changing the levels of $NO^{\cdot}$, $ONOO^-$ and H_2O_2 (17). Furthermore, recent results have showed ROS/RNS cross regulation of iNOS activity shifting the balance towards $NO^{\bullet}$ and $O_2^{\bullet-}$ production (18). This oxidant-mediated modification of iNOS function provides a mechanism for the regulation of iNOS enabling modulation of the amount of $NO^{\bullet}$ synthesis and the balance of $NO^{\bullet}$ and $O_2^{\bullet-}$ generation by the enzyme. These overlapping reactions provide a wide net of possible subtle cellular redox modifications that could fine tune physiological mechanisms of homeostasis.

2.2. Anti-oxidative defence and cellular redox state

The ADS is a functionally connected set of enzymes and low molecular weight components that have the physiological role of metabolising primary ROS components. The main ADS enzymes include primary SOD (both forms: Cu, Zn containing SOD or CuZnSOD or SOD1 and Mn containing or MnSOD or SOD2), CAT, glutathione peroxidase (GSH-Px) and glutathione reductase (GR). SOD converts $O_2^{\cdot-}$ to H_2O_2; CAT and GSH-Px decomposes H_2O_2 to H_2O. GSH-Px also converts lipid peroxides (LOOH) to corresponding alcohols (LOH) using gluthatione (GSH) as a reducing cofactor. These reactions require cellular GSH, which arises from the oxidised form GSSG. However, GSSG can be reduced by GR and this reaction uses NADPH as a reducing cofactor. In other words, anti-oxidant processes in cells consume GSH and/or NADPH. The cellular anti-oxidant capacity is supplemented with additional molecules that possess anti-oxidant and ROS scavenging and regulatory properties (peroxiredoxin, thioredoxin, metallothionein and glucose-6-phosphate dehydrogenase) and/or physiological functions (detoxification of secondary oxidative stress, xenobiotic detoxification and DNA repair). Thioredoxins (when reduced contain two –SH groups that form a disulphide when oxidized) bind to proteins and reduce protein disulphide bridges whilst oxidising themselves. Peroxiredoxins are a family of peroxidases that reduce H_2O_2 and organic peroxides by oxidising their own –SH groups which are subsequently reduced by thioredoxins. Metallothioneins are proteins rich in –SH groups, which can bind five to seven, metal ions via their association with cysteine-SH. It must be emphasised that ROS production and their elimination is a complex multi - step process and that the ADS composition is species and tissue specific and this must be given due consideration (19).

ROS and the ADS form parts of the cellular redox milieu. According to Scafer and Buettner the redox environment of a linked set of redox couples found in a biological fluid, organelle, cell or tissue is the sum of the products of the reductive potential and reducing capacity of the linked redox couples present (20). There are many redox couples in a cell that work together to maintain the redox environment. Changes in the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple appear to correlate with the biological status of the cell: proliferation, differentiation or apoptosis. Kirlin and co-workers showed that differentiation was associated with a dramatic oxidation of the GSH pool (21). The results indicated that a redox change of only 12 to 16 mV is sufficient to obtain significant increase in enzyme activity. Oxidation of the GSH pool is of a sufficient magnitude to contribute to redox-dependent regulation of gene expression either via S-thylation or via oxidation of vicinal dithiols, providing that the relevant E_{hc} values of control elements are in an appropriate range. The authors concluded that changes in the redox state could allow or disallow transcriptional activation of redox-sensitive genes, and this could be differentially regulated by factors affecting the GSH/GSSG redox state. Hence, physiological redox regulation represents the balance of intracellular thiol/disulphide redox states toward appropriate oxidative/reductive conditions and the adaptation to ROS can be assumed to be a chronic shift at the level of homeostasis.

2.3. Oxidative stress

The estimation of $O_2^{\bullet-}$ generation by mitochondria *in vivo* from $O_2^{\bullet-}$ production rates by isolated mitochondria is not possible and the extent of possible ROS-mediated oxidised cellular species is very broad (6). Exposure of proteins to ROS results in loss of parent amino acid residues, formation of unstable intermediates and the generation of stable oxidative modified products. If ROS production overwhelms the ADS, oxidative damage arises and oxidative stress can occur. Oxidative stress is a state when ROS production is above the ADS' capacity to eliminate them. According to numerous ROS-mediated processes, pathways that produce oxidative injury are multiple, complex and overlap. The sum of oxidative modifications is hard to measure and calculate and there is no universal single marker of oxidative stress. All together, the measurement of ROS production *in vivo* by the currently available techniques can not determine the exact ROS concentration in cells and the state of oxidative stress. The latter can be concluded indirectly from imperfect ROS measurements, the concentrations of pro-oxidative and/or anti-oxidative molecules, the changes in ADS activity and the quantities of oxidatively-damaged molecules. The measurement of all the above-mentioned parameters is difficult and is a very comprehensive task. Therefore, independent researchers describe separate changes in different model systems and on that basis a comprehensive picture of oxidative stress is built up. However, changes in the ADS are stated as a good indicator of homeostatic processes within the organism, the evidence that oxidative stress occurred *in vivo* and the cellular response to ROS and oxidative damages (10).

2.4. Redox cellular signalling

Mitochondrial ROS production is part of redox signalling connecting the regulation of mitochondrial function with the other cell compartments as well as the function of an integral cell (22). The key regulatory ROS molecule seems to be H_2O_2, due to its efflux from mitochondria and its ability to modulate the activity of proteins through the reversible oxidation of protein thiols (23). At physiological concentrations nitric oxide (NO), the $O_2^{\bullet-}$, and related ROS play an important role as regulatory mediators of vascular tone, oxygen tension monitoring, in respiratory control and erythropoietin production and signal transduction from membrane receptors in various physiological processes. Many of the ROS-mediated responses combine the protection of the cells against oxidative stress and the establishment of "redox homeostasis." Published data showing that redox changes in the cell affect various signalling pathways including transcription factors/activators (p53, AP-1 and NF-κB), tyrosine phosphorylation in proteins, src family kinases, JNK and p38 MAPK and the control of K^+ channels exist (24, 25, 26, 27, 28, 29). H_2O_2 induces the expression of the heme oxygenase gene and activates the transcription of NF-kB (30, 31).

3. MODELS FOR STUDYING COLD ANTI-OXIDATIVE DEFENCE RESPONSES

According to the general strategies to cope with cold there are three models for studying cold anti-oxidative defence: endotherms, heterotherms and ectotherms. Endotherms exposed to low temperatures elevate heat production followed by increased O_2 consumption which gives rise to more ROS in cells. In contrast, ectotherms decrease their metabolic rate and slow down their respiration during cold periods. In response to low temperatures heterotherms (hibernators) exhibit two characteristics: internal intrinsic (rhythmicity) and/or external (temperature and light cycle) closely linked to basic physiological regulatory mechanisms.

3.1. Endotherms

During cold exposure an acute stress reaction is replaced by mechanisms of adaptation. It has been known for long time that a 21-day period of cold exposure is sufficient for thermogenic adaptation and establishing basic physiological functions within a new level of thermogenic homeostasis (cold adaptation), including its own newly tuned physiological parameters (32, 33).

The acute cold stress reaction in rodents involves initial muscle shivering thermogenesis that is later replaced by a non-shivering state in the whole organism. Both processes are under neuronal and hormonal control and the sum of these reactions is the elevation of oxidative metabolism, heat production and oxygen consumption accompanied by elevated ROS production and a state of oxidative stress (34, 35, 36, 37, 38, 39, 40). However, cold exposure induces tissue specific responses according to its role in general thermogenesis. In rat brown adipose tissue (BAT) after 48h of cold exposure, uptake and catabolism of fatty acids and its beta-oxidation are accelerated (41). Furthermore, uptake and phosphorylation of glucose are both increased (35). Transcript levels of uncoupling proteins (UCPs) and B_1- and B_3- adrenergic receptors are elevated in BAT in cold conditions suggesting noradrenaline-mediated stimulation of UCP-1 expression during cold exposure. Moreover, UCP1 function in BAT is activated by fatty acids and the respiratory chain can afford extensive electron transfer (42). Acute cold exposure increases the mRNA levels of UCP2 and UCP3, and UCP3 protein in rat muscle (43, 44, 45). Fast twitch muscles seem to play a dominant role in acute adaptive thermogenesis in skeletal muscles via increased supply of fuel substrates (fatty acid and glucose) (46). In muscle, the increase in UCPs seems to be characteristic for acute cold exposure (up to 24h) but not long-term (47).

After long-term cold exposure a further increase in cytochrome oxidase activity, oxidative capacity, the rate of oxygen and food consumption and the demand for energy increase the metabolic rate and substrate turnover implying conditions of oxidative stress (48, 49, 50, 51). The level of oxidative stress is tissue specific according to its role in thermogenesis.

3.2. Heterotherms and ectotherms

Many intresting results have emerged from research into different forms of hypometabolism such as torpor and dormancy. These hypometabolic stages are part of survival strategies for a large number of organisms that cope with a variety of environmental stresses including low temperature. In addition to changes in the ADS, several other potential anti-oxidant mechanisms of cellular preservation and new insights into its control have emerged.

Hibernators, according to the season, can maintain body temperature and thermal homeostasis or hibernate. Hibernation involves actively lowering the body temperature and decreasing the metabolic rate according to seasonal and/or external temperature conditions. Physiological levels of the regulatory components are seasonally dependant and homeostatic setup is achieved by their different combinations. An example is the ADS activity in ground squirrels being highly seasonally affected and parallels other regulatory factors such as monoamine oxidase (MAO) activity (52, 53). However, it has been proposed that general thermoregulation of euthermic hibernators does not differ from that of non-hibernators and that a hibernating mammal, during euthermia, functions physiologically and behaviorally in the same manner as any non-hibernating mammal (54).

Hibernation is a closely regulated process. It has been proposed that antioxidant defence is increased in the BAT of ground squirrels at the onset of hibernation in order to protect tissue from ROS generated as a result of the intense metabolic activity sustained by this tissue during reawakening (55). The latter has been reviewed in more detail that allowed herein (10, 56, 57). Abrupt changes in the metabolic rate in ground squirrels arousing from hibernation, as well as in snails arousing from estivation, may also initiate conditions of increased ROS formation. In line with these ideas is that increased transcript levels of anti-oxidant enzymes including SOD, GSH-Px and glutathione-S-transferase (GST), as well as peroxiredoxins have been found in hibernating states (58, 59, 60). However, dynamic changes in anti-oxidants associated with hibernation vary with animal species and tissue studied (15, 61, 62, 63). Comparing the responses from diverse animals certain patterns have emerged. The most common was enhancement of the anti-oxidant defence. The increase in the baseline activity of key anti-oxidant enzymes, as well as 'secondary' enzymatic defence, and/or glutathione levels in preparation for an oxidative stressful episode arising from tissue reoxygenation seem to be the preferred evolutionary adaptation. A higher overall anti-oxidant capacity during anoxia/hypoxia is of relevance for species such as garter snakes (*Thamnophis sirtalis parietalis*) and wood frogs (*Rana sylvatica*) while diving freshwater turtles (*Trachemys scripta elegans*) appear to rely mainly upon high constitutive activities of anti-oxidant enzymes to deal with oxidative stress arising during tissue reoxygenation with reserves that some animal species might control post-anoxic ROS generation. Taken together, it seems that the cold defence strategy of these animals includes ADS elevation before the onset of cold conditions and intensive

thermogenesis. However, the ADS elevation is not uniform: in different tissues different ADS components are elevated (19, 55). Preparation for cold takes into account the role of specific tissue in hibernation and according to specificity reorganises ADS to allow the best protection at the lowest cost for overall thermogenesis. Recently, it has been shown that increased thermogenesis associated with arousal of the Arctic ground squirell results in tissue specific oxidative stress in BAT but not in liver. Moreover, torpor *per se* is largely devoid of oxidative stress, most likely due to suppression of oxidative metabolism (64).

Storey recently presented data that mRNA transcripts are regulated via inhibitory interactions with microRNA species during torpor, providing the first evidence of differential expression of mRNAs in hibernators (65). Current information about the regulatory mechanisms that govern gene expression during mammalian hibernation, in particular the potential role of epigenetic control (DNA methylation, histone modification, SUMOylation and the actions of sirtuins) in coordinating the global suppression of transcription has been reviewed. Long periods of deep torpor with brief arousal periods back to euthermia require coordinated controls that suppress and reprioritise all metabolic functions including global controls on both transcription and translation. Selected hibernation-specific genes are up-regulated under the control of specific transcription factors to support the torpid state; those that encode proteins involved in lipid fuel catabolism and in long term cytoprotection – anti-oxidants and chaperones. Global controls providing translational suppression also occur during hibernation including reversible phosphorylation control of ribosomal initiation and elongation factors as well as polysome dissociation. When taken together these mechanisms provide hibernators with multiple levels of regulatory controls achieving both global repression of gene expression and selected enhancement of genes/proteins that result in the hibernation phenotype.

Anti-oxidant defence during hibernation is in part controlled by NF-E2 related factor 2 (Nrf2) (66). Nrf2 protein in the heart was found to be elevated by 1.4-1.5 fold at multiple times during a torpor-arousal bout including during entry, long term torpor and early arousal. The Nrf2 levels returned to euthermic values when squirrels were fully aroused in interbout. Cu/Zn SOD levels have been shown to rise during entrance into hibernation by 1.5-fold and remain high during hibernation. The levels decreased significantly when squirrels were fully aroused from torpor, suggesting a role for Nrf2 in regulating the anti-oxidant defences needed for hibernation success. A similar mechanism occurs during dehydration/rehydration processes, which is accompanied by oxidative stress and ameliorated by enhanced anti-oxidant defences. The expression of Nrf transcription factor, GST, and aldo-keto reductase (AKR) analysed in the African clawed frog, *Xenopus laevis*, showed that metabolic responses to dehydration include the activation of the Nrf2 transcription factor and selective up-regulation of genes under Nrf2's control via the anti-oxidant response element (ARE) that is present in the promoter regions of GST and AKR genes (67).

Insects possess different strategies to cope with the cold (68, 69). A comparison of the freeze-tolerant European corn borer *Ostrinia nubilalis* and the freeze-intolerant Mediterranean borer *Sesamia cretica* exposed to low temperature (-3^{o}C) revealed that *Ostrinia* larvae elevated ADS components, while in *Sesamia*, changes in the ADS can be defined as a cold stress response. A further decrease in temperature exceeds stress resistance capacities resulting in death (70). Freeze-tolerant *Ostrinia nubilalis* species respond to cold by elevating the level of anti-oxidant defence. This suggests a mechanism for preparing the body for free radical attack during the thawing and re-warming process, as well as the preservation of redox homeostasis, regulation of physiological concentration of redox molecules (ascorbate, GSH, NADPH, H_2O_2 and NO) and promotion of the pentose phosphate pathway (PPP). In cold-hardy insects the flux through the PPP is critically important for generating the reducing equivalents (NADPH) needed for the synthesis of polyol cryoprotectants. It has been shown that for the synthesis of glycerol from glycogen, 86% of the total carbon flow must be routed through the PPP to generate the required reducing power. Furthermore, glycerol, ethylene glycol and trehalose are accumulated during the winter period in freeze-tolerant insect species. These compounds act as both anhydroprotectors and anti-freeze compounds and are believed to be free radical scavengers. Recent results demonstrate that intracellular phosphorylated forms of fructose have scavenging properties and could be taken into account regarding overall anti-oxidant capacity (71). The important compound in insects is ascorbate. In insects an ascorbate enzymatic recycling system which yields reduced ascorbate exists. However, dehydroascorbate (oxidised ascorbate) at a physiological pH is unstable with a half-life of a few minutes It may be rapidly metabolised through diketogluconate to 5-carbon intermediates which could enter the PPP. In *Ostrinia nubilalis* low temperature favours the catabolism of sugars via the PPP generating reducing power (NADPH) for polyol synthesis. An elevation in the activity of the PPP enzymes is especially pronounced in early diapause. The level of glycerol and the activity of key enzymes of the PPP support the connection between this metabolic pathway and the anti-oxidative system and the notion that the ADS in larvae of *Ostrinia nubilalis* is closely connected with metabolic changes characteristic of diapause and cold hardiness. Studies on the level of ADS enzymes in diapausing *Ostrinia* larvae exposed to low temperature (8^{o}C and -12^{o}C) revealed different responses to cold in November and February (72). There is a dual role of the ADS: in November the ADS is active towards protection and promotes higher PPP activity, while in February the role of the ADS seems to be focused on the preservation of reduced ascorbate underlining the importance of reduced ascorbate in the physiology of this insect species. When studying the role of the ADS during insect cold resistance both the developmental stage and the age have to be taken into account since the response seems to be different according to the developmental programme

(73, 74). Moreover, in mitochondria of diapausing *Ostrinia* larvae the pattern of ADS enzymes seems to parallel changes in energy production and O_2 consumption and protect against oxidative stress (75).

A recent result concerning the arctic springtail *Onychiurus arcticus* showed that genes for anti-oxidants are among numerous genes involved in the survival in polar extreme conditions (76). *Onychiurus arcticus* regularly survives extreme cold conditions by reducing the amount of water in its body to almost zero: a process that is called "protective dehydration". A number of genes participating in the process were identified, with particular interest in the trehalose and glycogen pathways, aquaporins: 6-transmembrane domain proteins involved in H_2O transport and anti-oxidants. The highest number of anti-oxidant clones was present in both the desiccating and desiccated libraries and from the percentages of clones present in each library for each anti-oxidant (the major components would appear to be GSH, CAT and H_2O_2).

The important role of SODs in cold defence is supported by results in cold-induced longevity (77). Mutational inactivation of each SOD isoform during cold-/hypothermic-induced longevity (CHIL) significantly reduces lifespan extension in worms by CHIL, suggesting that CHIL requires a specific genetic programme beyond a simple reduction in metabolic rate. Furthermore, CHIL paradoxically increases lifespan while reducing resistance to oxidative stress, further disassociating oxidative stress resistance and lifespan.

4. COLD ANTIOXIDATIVE DEFENCE RESPONSES

The response of organisms to low environmental temperature depends on both the length of exposure and the degree of coldness. ADS studies in rats showed that the time required for ADS cold adaptation was longer than for thermogenic adaptation and was also tissue specific (78). The ADS is thought to be a supporting system in environmental and programmed adaptations to low temperatures intercalated into physiological mechanisms of homestasis (79). In endotherms, the ADS fights against ROS in acute cold conditions, but oxidative damages occur, suggesting it is overwhelming. In prolonged environmental low temperature exposure and physiological addaptation, the ADS establishes higher levels of activity in order to protect against sustained oxidative pressures to preserve redox cellular signalling.

4.1. Short-term cold exposure

An elevation of oxidative metabolism, heat production and oxygen consumption increase ROS levels and create a state of oxidative stress (28, 29, 33). In rat liver mitochondria the rate of H_2O_2 release and the amount of lipid peroxides and protein carbonyls (products of oxidatively-modificated proteins) gradually increases during cold exposure as ROS production completely overwhelms mitochondrial anti-oxidant capacity (28). The overall anti-oxidant capacity measured by an enhanced luminescence technique gradually diminishes during cold exposure in rats (80). In addition, a gradual increase in the susceptibility to oxidants and possible $OH^{\cdot}$ production was evident. The ADS responds to oxidative pressure during acute cold stress conditions, but not to such an extent to prevent oxidative stress and damage (81, 82, 83). In the most active tissues during short-term cold exposure the ADS level is mediated by enzyme modifications and changes in its activity rather than its protein synthesis. Modulators of ADS enzyme activities could be ROS themselves, but others can also be included, especially NO and reactive nitrogen species. Protein synthesis is an ATP-consuming process and requires time. Short-term cold exposure is characterised by high ATP and NADPH consumption and protein synthesis *de novo* seems too costly regarding acute reactions. Also, the role in maintaining redox state of the cells is an important part of ADS system. This implies that the early acute phase of cold exposure is accompanied by a significant depletion of redox equivalents. It is questionable if the ADS has the capacity to neutralise elevated ROS because, in addition to GSH depletion, high-energy demands (elevated ATP consumption) are also present. However, the sum of physiological factors contributes to oxidative stress associated with short term cold exposure is not definite and some others factors could be involved (NO, adrenergic stimulation and catecholamines). There are data that uncoupling proteins (UCPs) influence and are regulated by mitochondrial ROS production (84, 85, 86, 87, 88). Mild uncoupling has been suggested to be a defence strategy against ROS since UCPs reduce membrane potential and Ca^{2++} mitochondrial accumulation, both generators of ROS production (89, 90, 91, 92, 93). However, it has also been noted that neither ROS nor ROS products activate UCPs, as well as that UCPs have no role in protection against oxidative damage (94). According to differential tissue expression of individual UCPs, these processes have tissue-specific relative roles. Furthermore, the expression of UCPs is under the influence thyroid hormones (95). The sum of the responses to cold is the establishment of a new set point of homeostatic regulators with different a ratio of individual ROS production. The role of UCPs in cellular generation and/or protection from ROS remains to be clarified.

A comparative study of short-term exposure of rats and euthermic ground squirrels to cold showed that ground squirrels responded to low temperatures faster. In ground squirrels physiological responses regarding utilisation of glucose and free fatty acids during this acute phase of cold exposure suggested differential temporal physiological responses, although the underlying mechanisms could be the same (83). In winter, ground squirrels hibernate with short periods of activity. When animals are kept warm (maintained at thermoneutrality) in winter, they are euthermic and avoid hibernation. Euthermic ground squirrels in winter have the lowest level of the ADS compared to the other seasons confirming that the ADS accompany basal metabolic conditions (19, 52, 79, 83).

4.2. Long-term cold exposure

There are data showing several similar effects of cold exposure and triiodothyronine (T_3) treatment on ADS enzymes (96, 97, 98). Some of the changes in the ADS

enzyme composition during cold exposure are mediated by thyroid hormones. Thyroid hormone-mediated processes are elevated during long-term cold exposure appearing as a hyperthyroid state. Venditti and colleagues have shown that the hyperthyroid functional state induced in mammals by cold exposure, like experimental hyperthyroidism, led to enhancement of their basal metabolic rate, which reflects an increase in the cellular respiration of target tissues including BAT, liver and cardiac and skeletal muscles (35, 99, 100). There are data indicating that in hyperthyroid tissues increased ROS and RNS production, as well as the decrease in antioxidant capacity and susceptibility to oxidative damage occurs (101). A side effect of an enhanced level of electron carriers, by which hyperthyroid and tissue exposure to cold increase their metabolic capacities, is increased mitochondrial ROS generation (102, 103). Thyroid hormone causes many of the biochemical changes underlying tissue oxidative damage and the ADS response. It is still not clear if ADS responses are the effect of the thyroid hormone action or ROS-mediated cellular regulation.

Three-week cold exposure is accompanied by GSH tissue depletion suggesting a state of chronic redox disequilibrium (51). This observation was more profound in the heart, liver and small intestine, since a decrease in GR activity, confirming the deficiency of reducing equivalents, was also recorded. In tissues where substrate turnover is high, such as the kidney and small intestine, the need for redox equivalents is compensated by elevated ascorbate. It has been postulated that ascorbate and GSH are important cellular redox cofactors with overlapping physiological roles (104).

4.3. Tissue specificity

During cold exposure the ADS strives against conditions of oxidative stress in a tissue-specific manner (78). Initial muscle shivering thermogenesis is accompanied by elevating the ADS in skeletal muscle while in cold-adapted animals the ADS is attenuated due to a shift to non-shivering thermogenesis in muscles (105). In BAT, which is the main thermogenic tissue in cold-adapted animals, the ADS is elevated after long-term cold exposure (78). The ADS composition in BAT is changed according to the newly established metabolic profile in the tissue (106). These results suggest that in thermogenic tissues the ADS support environmental and programmed adaptations to low temperatures (79). In rat brain acute cold-exposure induces anti-oxidative defence elevation responses that are mainly influenced by adrenergic stimulation, catecholamine metabolism and local ROS-mediated processes (107, 108). The elevation of the ADS in tissues is at the level of individual ADS components, specific ROS profiles and general metabolic and functional demands for separate tissues. This demonstrates that the ADS is a dynamic, multi-level physiological system incorporated in the mechanisms of the regulation of homeostasis.

5. CELLULAR REDOX SIGNALLING AND REGULATION OF ANTIOXIDATIVE PROCESSES DURING COLD EXPOSURE

ROS involvement and the role of the ADS in organisms subjected to low tempreatures are features intercalated into physiological mechanisms of homestasis. The exact mechanisms behind ROS and ADS regulation have not yet been fully defined and are the subject of many ongoing intruiging scientific investigations. Possible mechanisms include those at different levels: cellular, tissue and integrative. Since ROS are generated as a normal part of aerobic metabolism (mitochondrial oxidative phosphorylation), the control of metabolic rate and oxygen consumption influences ROS production. The existence of a feedback loop between ROS and H^+ leakage has been suggested (109). ROS and $NO^{\cdot}$ can inhibit or change the activity of several metabolic and ADS enzymes leading to different levels and types of ROS production. ROS can activate and inactivate transcription factors, membrane channels and metabolic enzymes and regulate calcium-dependent and protein phosphorylation signalling pathways. The proposed mechanism by which ROS integrate into cellular signal transduction pathways is via oxidation and reduction of thiol proteins. Detailed mechanisms behind the oxidation of regulatory thiol proteins still need to be elucidated (110). Anti-oxidative defence is a NADPH and/or GSH consuming process and it influences the amount of cellular redox equivalents. This in turn could define the cellular redox environment and cell fate allowing (or not) transcriptional activation of redox-sensitive genes. In cold exposure the distribution of the ADS is tissue specific depending on the metabolic profile of the tissue in question and its involvement in a more broad sense of the internal *milieu*.

Cold defence responses in the animal kingdom exhibit very broad strategies with a high dependency on anti-oxidants. Recent results from hibernators have highlighted multiple levels of regulatory control, both global repression of gene expression and selected enhancement of genes/proteins, representing the hibernation phenotype. The same is true for several forms of dormancy. The similarity of the ADS response in hetero- and ectotherms suggests some common mechanisms underlying the ADS in physiological processes and whole-body regulation. Furthermore, the ADS is an internal system integrated into a number of cellular and physiological regulatory mechanisms with a role to serve and protect homeostasis. Such flexibility is subject to a number of regulatory modifications that allow it to respond to internal and environmental changes.

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