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Cold Adaptation as a Means of Increasing Antioxidant Protection

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We present here a review of the literature analyzing the link between the physiological mechanisms of cold adaptation and cold-induced prooxidant and compensatory antioxidant processes. Cold adaptation has been shown to increase the number and activity of mitochondria to support increases in ATP consumption; this leads to increased generation of reactive oxygen species (ROS), as mitochondria are among the main sources of ROS in physiological conditions. Studies in recent decades have shown that protein PGC-1α – one of the main regulators of mitochondrial biogenesis – also affects the synthesis of antioxidant enzymes, thus controlling the mitochondrial generation of reactive oxygen species. At the whole-body level, systematic cold stimulates intrinsic protective resources by enhancing oxidative processes, which in turn initiates activation of the antioxidant systems and increases the overall resistance of the body to stress factors of different types.

Keywords: cold adaptation, reactive oxygen species, antioxidant system, nonspecific resistance.

Cold Adaptation, Mitochondrial Growth, Increases in the Concentration of Reactive Oxygen Species, and Strengthening of the Antioxidant System. Many studies addressing the main mechanisms of cold adaptation have shown that the aim of physiological changes in these conditions is to increase heat production and decrease heat loss. The main sources of additional heat energy include muscles and brown fat. However, in humans (in contrast to laboratory rodents – which are the most widely used system for studies of thermoregulation), the contribution of brown fatty tissue is small because of the small quantity present. The main increase in heat production is achieved by increasing contractile activity particularly of oxidative muscle fibers (muscle shivering and thermoregulatory muscle tone) and changes in the energetics of muscle contraction [8, 17, 67, 70, 77, 94]. Increases in the rate of mitochondrial respiration are seen in cold adaptation, with a simultaneous decrease in the coupling of oxidation and phosphorylation due to an increase in the proton permeability of the inner mitochondrial membrane, which leads to an increase in the proportion of energy from the proton potential dissipated as heat [8, 17, 24, 38, 47, 60, 67]. It should be noted that these changes are seen not only in muscles, but also in liver [25], which, according to data reported by Jansky [47], can achieve an increase in heat production by up to 25%. Apart from an increase in the permeability of mitochondrial membranes, membrane permeability also increases in other cellular organelles and ion pump activity increases to compensate for the corresponding ionic leakage, which is also accompanied by an increase in heat release [28, 35, 45, 66, 76, 80, 82]. ATP-dependent membrane transport affects all the cells in the body, and as a significant proportion of the energy in this situation is dissipated as heat, it is also an important source of heat production. Provision of a sufficient quantity of ATP in conditions in which the proportion of mitochondrial energy dissipated as heat increases is ensured by increases in the number of mitochondria and their functional activity [5, 25].

Members of a family of nuclear receptors – peroxisome proliferator-activated receptors (PPAR) – have been actively studied in recent years. These studies have shown that one of the common coactivators of the PPAR family – PPARγ 1-α-coactivator (PGC-1α) – plays a major role in energy provision to skeletal muscles, the myocardium,

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and fatty tissue. PGC-1α was first identified by Puigserver et al. [73] in brown adipocytes as a cold-induced transcription coactivator of PPARγ. Further studies showed that it is a key regulator of mitochondrial biogenesis and oxidative metabolism in skeletal muscles, myocardium, brown fatty tissue, and other cell types [27, 40, 57, 62, 66]. Many authors have reported increased expression of PGC-1α, and an increase in the proportion of oxidative fibers has been seen on cold adaptation [33, 50, 70, 77]. Bruton et al. [33] studied the muscles of the plantar part of the foot (flexor digitorum brevis) in rats, which is not involved in muscle shivering on exposure to cold, and showed that cold adaptation significantly increased PGC-1α expression (by 50%) and citrate synthase activity, which is evidence of a significant increase in the number of mitochondria. Furthermore, an increase in the free calcium concentration in the cytoplasm is seen both at rest (by 50%) and on electrical stimulation of contraction (by 40%), along with a four-fold increase in the membrane permeability of the sarcoplasmic reticulum for calcium ions.

Intensification of oxidative processes (contractile activity of oxidative muscle fibers and increased rate of mitochondrial respiration) leads to increases in the concentration of reactive oxygen species (ROS), as some proportion of the oxygen passing through the mitochondrial electron transport chain is converted into superoxide anion radicals, giving rise to other ROS [1, 18]. In addition, increases in the intracellular calcium ion concentration can lead to increased mitochondrial ROS generation [43, 98] and activation of a series of ROS-forming enzymes [16]. In physiological conditions, the greatest contribution to the increase in ROS concentration is made by the mitochondrial respiratory chain, the greater proportion of ROS being produced by complexes I and III of the electron transport chain. Exact determination of the quantity of ROS in mitochondria is difficult because of the need to retain the structural integrity of these organelles during isolation. In addition, mitochondria from different organs show significant differences in terms of the activity of electron-transporting structures and antioxidant contents [90]. This leads to significant differences in experimental results. Data from some researchers using normal conditions for cell functioning indicate that ROS are formed in mitochondria at quantities of 0.15% of the oxygen consumed [88], while others have assessed ROS production at a level of 4–5% of the absorbed oxygen [100]. Apart from mitochondria, other intracellular sources of ROS are peroxisomes, xanthine oxidase, myeloperoxidase, uncoupled NO synthase, cyclooxygenase, lipoxygenase, cytochrome P450, and NADPH oxidase.

A significant increase in the ROS concentration leads to oxidative stress (lipid peroxidation in cell membranes, cleavage of DNA strands, oxidation of membrane-bound proteins, inactivation of enzymes, oxidation of carbohydrates).

In addition, many studies have shown that in physiological conditions, ROS are involved in many key regulatory cellular mechanisms, the control of ion transport, and renewal of cell membranes, as well as hormone and prostaglandin biosynthesis and degradation of xenobiotics [7, 16, 18]. The main physiological functions of ROS are: 1) involvement in the synthesis of messenger-type molecules (e.g., eicosanoids: prostanoids and leukotrienes) and iodothyronines; 2) oxidation of damaged molecules for their subsequent recycling; 3) they have signal functions (ROS-dependent intracellular systems transferring external signals to the cell nucleus, with subsequent initiation of protein synthesis) [18, 23, 75].

The most stable ROS is the hydrogen peroxide molecule (H_2O_2). Being electrically neutral and having good solubility in water, the H_2O_2 molecule easily crosses biological membranes and can migrate into cells and tissues [23]. However, the intracellular concentration is 8–10 times lower than the extracellular concentration, because cells contain the antioxidant enzymes catalase and peroxidase, which degrade hydrogen peroxide [16].

Repeated restriction of ROS generation with subsequent synthesis of protective systems provides a mechanism increasing the resistance of the body on adaptation to cold [18], physical loads [19, 72], cold [84], and hypoxia [18, 36]. Sazontova et al. proposed the concept that ROS are involved in forming the nonspecific resistance of the body in conditions of periodic actions of external factors [18]. Redox signaling, without any specific receptors, supports cellular responses to hypoxia, oxidizers, and reducers. Furthermore, mediators whose actions operate via hormone or cytokine receptors also activate nonspecific redox signaling in receptor cross-reactions, which is the basis of the cross-effects of adaptation in which adaptation to one external factor increases the body's resistance to the actions of another factor.

In physiological conditions, active ROS generation in the body is accompanied by increased antioxidant protection. The antioxidant protection of mitochondria is of particular interest, as mitochondria are one of the main sources of ROS in cold adaptation, as in other physiological conditions. The superoxide concentration in the mitochondrial matrix in the stationary state was 5–10 times higher than that in the cytoplasm [34].

Inactivation of superoxide in mitochondria is mediated by manganese-dependent superoxide dismutase (SOD2) which dismutases superoxide to form hydrogen peroxide, which is detoxified by glutathione peroxidase. The quantity of catalase in mitochondria is low, while muscle mitochondria do not contain catalase at all [16]. Borras et al. [32] compared mitochondria from female and male rats and showed that gene expression and SOD2 and glutathione peroxidase activities in female liver mitochondria were twice as high as in male mitochondria, such that hydrogen peroxide production in male liver mitochondria was 80% greater and the content of reduced mitochondrial DNA bases (8-oxo-2-deoxyguanosine) was four times greater. Ovariectomy eliminated differences; the differences recov-

ered on the background of treatment of post-ovariectomy females with β-estradiol [32, 91], pointing to a strong interrelationship between the elements of the antioxidant system – the enzymes superoxide dismutase and glutathione peroxidase – and sex hormones (sex hormones, like catecholamines, are phenolic compounds, which explains their high antioxidant activity) [61, 58, 83, 99]. Furthermore, various studies have shown that the rates of formation of superoxide and hydrogen peroxide in mitochondria in different animal species are inversely proportional to the mean duration of life of members of these species [53, 59]. Apart from superoxide dismutase and glutathione peroxidase, glutathione (GSH) is a significant component of the antioxidant system of mitochondria, protecting or restoring damage formed during aerobic metabolism [9, 16]. Glutathione peroxidase activity and the rate of hydrogen peroxide utilization are directly dependent on the intracellular concentration of reduced glutathione [14]. Glutathione in its reduced form can function as an antioxidant by many means: chemical interaction with singlet oxygen, superoxide, and hydroxyl radicals or direct degradation of free radicals; stabilization of the membrane structure by transportation of acylperoxides formed by lipid peroxidation (LPO). GSH is a coenzyme for a series of enzymes whose activities are based on changes in the redox potential of glutathione. Changes in the ratio of reduced and oxidized glutathione (GSH/GSSG) are of particular interest – this is one of the main indicators of redox status and an important factor in signal transduction [49]. In addition, GSH plays an important role in the repair of damaged DNA molecules. Glutathione is not an especially effective radioprotector for DNA molecules exposed to x-rays, but regulates the mechanism reversing damage to DNA molecules [74]. Glutathione synthesis does not take place in mitochondria; 45–50% of the GSH in the mitochondrial matrix arrives by means of transporters located on the inner mitochondrial membrane and working against an electrochemical potential gradient [9], which makes this process ATP-dependent.

In the framework of analysis of the influences of cold on the antioxidant system, apart from mitochondria, which undergo significant growth in muscles on cold adaptation, the antioxidant systems of erythrocytes and plasma are of significant interest, as the overall state of the body's antioxidant system is assessed in most studies in terms of measures of these systems in the blood.

The glutathione system, including reduced glutathione and glutathione-dependent enzymes, plays a special role in protecting erythrocytes, as glutathione peroxidase prevents the formation of methemoglobin from hemoglobin in the presence of hydrogen peroxide; the glutathione concentration in erythrocytes is 100 times greater than that in plasma [16]. Apart from the glutathione system, the enzyme catalase, which degrades hydrogen peroxide, plays an important role. In humans, the greatest quantities of catalase are present in erythrocytes, liver, and kidneys [16].

The most significant endogenous components of the antioxidant system of the plasma consists of extracellular superoxide dismutase (SOD3), glutathione peroxidase, glutathione, and ceruloplasmin and uric acid, which are transition metal chelators [16].

Apart from endogenous antioxidants (AO), antioxidants ingested with the food are also significant, these including fat-soluble AO – tocopherols and carotenoids, which protect biological membranes – and water-soluble AO – ascorbic acid, rutin, and others. The oxidized forms, such as the fat-soluble AO tocopherol and ubiquinone, can be reduced by the water-soluble AO ascorbic acid; ascorbic acid also has a role in maintaining glutathione in the reduced state [16]. α-Tocopherol contents in mitochondria isolated from different mouse organs correlate negatively with the production of superoxide anion radicals, pointing to its protective and, perhaps, regulatory role [2]. In addition, high contents of α-tocopherol are found in species and populations of animals living in cold conditions and this increases with increases in the unsaturatedness of membrane lipids [2, 10].

Studies reported by Kolosova et al. showed that administration of tocopherol in the cold leads to decreases in membrane lipid peroxidation both in animal experiments [11] and in human studies [12, 13]. In addition, the use of food supplements containing exogenous antioxidants is not always effective as they can induce the body's intrinsic antioxidant system [18].

Studies conducted in the last decade have shown that the expression of the *PGC-1α* gene increases in conditions of oxidative stress and that PGC-1α protein induces increases in antioxidant enzymes [27, 44, 75, 86, 88, 91]. St Pierre et al. [88] showed that PGC-1α knockout mice had decreased levels of expression of the genes encoding all three types of superoxide dismutase and glutathione peroxidase and that these mice were more sensitive to oxidative stress. Conversely, increased PGC-1α expression increases antioxidant protection, as it induces increases in the expression of manganese superoxide dismutase, glutathione peroxidase, and catalase [75]. Furthermore, PGC-1α increased expression of uncoupling proteins UCP2 and UCP3, promoting reductions in ROS generation by mitochondria [75].

Thus, PGC-1α – one of the main regulators of the biogenesis of mitochondria (one of the main sources of reactive oxygen species) – also regulates the generation of reactive oxygen species.

Apart from antioxidants directly in contact with ROS, a so-called secondary antioxidant protection system is also sometimes defined [37], which supports reversal of damage occurring in cells as a result of interactions with ROS. This includes the large number of DNA repair systems, specific proteolytic enzymes, and macrophages, effectively collecting oxidized lipoproteins in plasma using "scavenger receptors."

The Training Role of Cold. Cold, like any stress, is known to be accompanied not only by a specific response,

but also by a nonspecific response. Increases in the level of reactive oxygen species constitute one sign of this nonspecific response, which is mediated by redox signaling and induces the synthesis of protective systems: antioxidant protection enzymes, protooncogenes, heat shock proteins, specific chaperones for membrane-bound proteins, and others [18, 48]. Siems et al. [78] showed that an acute cold stimulus (winter swimming) increases the concentration of reduced glutathione in erythrocytes, as well as the proportion of glutathione in the oxidized state, which is a consequence of the increased ROS level in the body. Furthermore, prolonged cold adaptation (systematic winter swimming) leads to an increase in the baseline concentration of reduced glutathione and a decrease in the cold stimulus-induced relative increase in oxidized glutathione. In addition, erythrocytes in those enjoying winter swimming have higher baseline concentrations of the antioxidant enzymes superoxide dismutase and catalase [78, 79].

The nonspecific response to stress is the basis of so-called preconditioning, which is a procedure in which a fairly strong stimulus acts on the body without inducing structural/functional damage. The result of this treatment is that mechanisms of resistance to similar stimuli or different stimuli – cross-activation – form in the body [3, 6, 15, 21, 51].

The cross-effects of cold adaptation have been demonstrated in reports from many researchers. Experiments on rats with deoxycorticosterone-salt hypertension showed that systematic exposure to cold (0–4°C for 6 h) for four months had an antihypertensive effect [4]. Lunt et al. [65] showed that periodic cold increases resistance to hypoxia. Continuous cold, conversely, decreased it [41]. A possible cause of these differences is the fact that prolonged cold adaptation increases oxidative metabolism and increases the proportion of lipid metabolism in the overall energy supply to the body, which makes the body more sensitive to oxygen deficit. However, adaptation to continuous cold leads to increases in resistance to radiation. Levan et al. [57], Ghys [42], and Barlow and Sellers [31] showed that preliminary adaptation to cold increased the survival of animals subsequently exposed to high-dose x-ray irradiation. The cytotoxic and cancerogenic actions of ionizing radiation on living organisms are directly related to the generation of OH radicals during the radiolysis of water, which applies to the greater proportion of cells and multicellular organisms. Increases in the antioxidant and repair systems during cold adaptation evidently promote reductions in oxidative stress, which increases survival in animals. Akhalaya et al. [1] showed that adaptation of animals (mice) to swimming in cold water led to an increase in resistance to the actions of high doses of adrenaline. Maximal antioxidant activity and resistance to adrenaline shock were seen one day after swimming.

In recent years, cold exposure has increasingly been used for medical purposes. Over more than three decades, transient cooling at ultra-low temperature in a cryosauna has been used in medical centers to treat pain in joint diseases, acute and chronic sport traumas, and rehabilitation after competitions [29, 40, 89, 93, 95, 97]. Exposure to ultra-low temperatures was first used by the Japanese rheumatologist Tosimo Yamauchi to treat joint diseases at the end of the 1970s [97]. Data from numerous investigators [30, 39, 52, 62, 71] show that systematic cooling in a cryosauna leads to an increase in the antioxidant potential of the body and decreases in creatine kinase and lactate dehydrogenase induced by high-power physical loading. Furthermore, the lipid profile changes [61].

Wozniak et al. [96] reported an analysis of delayed reactions to single exposures to ultra-low temperature (–120°C) showing that the superoxide dismutase and glutathione concentrations in erythrocytes increased. Similar results were obtained by Lubkovska et al. [62], which demonstrated that single episodes of cooling in a cryosauna (–130°C) increased the glutathione concentration and glutathione peroxidase and glutathione reductase activities in erythrocytes, but reduced glutathione transferase activity; the superoxide dismutase concentration increased slightly immediately after cooling but decreased significantly on following days compared with baseline. The authors came to the conclusion that transient cooling at ultra-low temperature in the cryosauna mainly affected the glutathione-linked antioxidant system. Increases in the antioxidant system were induced by increases in the activity of oxidative processes: both studies demonstrated significant increases in measures characterizing oxidative stress in both erythrocytes and plasma (TOS [22], conjugated dienes and TBARS [96]). A long-term study reported by Lubkovska et al. [63] included analysis of 20 daily exposures in the cryosauna and showed that the overall cycle of cooling was followed by an increase in the basal superoxide dismutase level but decreases in the levels of reduced and oxidized glutathione and glutathione peroxidase. In the mid-part of the cycle (by day 10 of cooling), there were significant increases in both forms of glutathione. The catalase concentration also increased in the mid-part of the cycle but returned to baseline by its end (by day 20 of cooling). There were significant changes in the plasma uric acid concentration: it decreased in the mid-part of the cycle and increased by its end; the ceruloplasmin level also decreased significantly at the end of the cycle. 8-Isoprostane, which forms as a result of free-radical oxidation of phospholipids in cell membranes, provides a marker for oxidative stress. Increases in this parameter were recorded immediately after the first cooling, and the baseline value (before cooling) at mid-cycle was, on average, more than two-fold greater than the initial value, though there was no change in this indicator in the second half of the cycle. Thus, this study demonstrated that daily cold exposure at ultra-low temperature for 20 days leads to a stable increase in the level of lipid peroxidation (8-isoprostane). This increased level formed after 10 daily exposures and remained essentially unaltered throughout the following 10 daily cold exposures. In the second half of the cycle, there were sig-

nificant changes, in both directions, in both enzymatic and nonenzymatic antioxidants.

Rather different results were obtained by Miller et al. [69], who used a cycle of 10 cold exposures at ultra-low temperature (–130°C), divided into two parts of five daily exposures separated by a two-day break. This study analyzed the dynamics of total antioxidant status (TAS), the uric acid concentration, and erythrocyte superoxide dismutase activity, as well as the dynamics of thiobarbituric acid-reactive substances (TBARS), a measure of oxidative stress. After the end of the whole cycle of 10 cooling sessions, the uric acid concentration, erythrocyte superoxide dismutase activity, and total antioxidant status increased significantly, while TBARS decreased. The differences in the results of these studies may be due to the fact that the studies reported by Miller et al. [69] included a two-week break between the sets of five daily cold exposures, which was evidently sufficient for repair and adaptive changes induced by cold stress. As the de novo synthesis of antioxidant enzymes and glutathione is an ATP-dependent process, it is logical to propose that it will occur more effectively when there is sufficient ATP, i.e., between coolings. The studies reported by LeBlank and Labrie [55] on rats showed that formation of resistance to intense cold required at least 24 h, which is also consistent with results reported by Akhalaya et al. [1], showing that the maximum antioxidant activity was seen one day after cooling. Considering that the rate of metabolism in humans is lower than that in rats and mice, the required time interval is evidently longer. This is supported by results from analysis of the cold adaptation of winter swimming enthusiasts. Lubkovska et al. [64] showed that adaptation to winter swimming by swimming 2–3 times a week during the winter months was followed by significant reductions in measures of oxidative stress such as 8-isoprostane (by an average factor of 2), along with a significant increase in the concentration of reduced glutathione with a reduction in the quantity of oxidized glutathione and increases in the superoxide dismutase, catalase, glutathione reductase levels and decreases in the glutathione peroxidase level. These data are consistent with the earlier results of Siems et al. [79] showing that winter swimming enthusiasts have a higher basal erythrocyte glutathione concentration and higher superoxide dismutase and catalase activities.

Despite certain differences in study results, all are united in the opinion that the glutathione antioxidant system is significantly activated in response to cold. This may be associated with the fact that in studies on the effects of cold, the analysis included only those measure of the blood antioxidant system, while the glutathione system plays a particularly important role in protecting erythrocytes [16]. In addition, the glutathione system is one of the main systems (apart from manganese SOD) in mitochondria; as already noted, cooling activates mitochondrial respiration, which unavoidably increases the quantity of ROS and, thus, antioxidants. However, it should be noted that increases in mitochondrial respiratory activity on cooling are seen particularly in skeletal muscle myocytes and hepatocytes, whose antioxidant status in vivo is significantly more complex to analyze. Hydrogen peroxide molecules, which are electrically neutral, can easily migrate to the blood and other tissues, leading to compensatory activation of antioxidant enzymes in these locations. This may be the cause of the increases in glutathione peroxidase and catalase activities recorded in erythrocytes, where they degrade hydrogen peroxide.

Apart from cooling-induced increases in the activity of antioxidant enzymes and glutathione and uric acid levels, many studies have demonstrated increases, by factors of 2–4, in the noradrenaline concentration [8, 17, 81] – noradrenaline, on the one hand, is a "mediator of thermogenesis" in cooling and, on the other, is an antioxidant [16, 58, 83, 99]. Studies reported by various investigators [20, 46, 56] show that transient cooling at ultra-low temperature (–110 and –70°C) was followed by significant increases in the blood noradrenaline concentration (more than 200% [46, 56] and 50% [20]), the adrenaline concentration not undergoing any change. Similar results (a five-fold increase in the noradrenaline concentration without any significant change in the adrenaline level) were obtained by Sramek et al. [85] and Leppaluoto et al. [56] on analysis of catecholamine concentrations in blood tests after cold immersion (14°C for 1 h [85] and 0–2°C for 20 sec [56]).

The increase in the antioxidant system in response to cold demonstrated in many studies initiated a series of investigations for analysis of the effects of cooling at ultra-low temperature on oxidative stress induced by severe physical loads. Studies reported by Wozniak et al. [96] compared two groups of intensely trained, highly experienced sportsmen, one of which used daily cooling in a cryosauna, and showed that after 10 days of such training the group of sportsmen using cooling had high levels of antioxidant protection enzymes (superoxide dismutase and glutathione peroxidase), though measures of oxidative stress, i.e., conjugated dienes and TBARS, were higher. Similar results were obtained by Mila-Kierzenkowska et al. [68]. These authors concluded that there was an increase in antioxidant protection as a result of using cooling at ultra-low temperature during the high-intensity training cycle. However, increases in the level of antioxidants could not be interpreted as increases in cell protection, as the root cause of this increase is induction of ROS. It should be noted that the high antioxidant level can be accompanied by a low level of free-radical processes – in this case, the cell will have additional antioxidant protection – and a high level of ROS – in this case both compensation of free-radical processes and the absence of the same can be seen [18].

Thus, studies conducted in the last decade lead to the conclusion that systematic cold treatments activate different components of the antioxidant system and prevent the development of oxidative stress induced both by cold itself and by other types of stressors. PGC-1α, one of the main

regulators of mitochondrial biogenesis (the main sources of ROS in cooling), also affects the synthesis of antioxidant enzymes, thus controlling the generation of reactive oxygen species by mitochondria. Moderate stress stimulates the body's intrinsic protective resources by increasing oxidative processes, which in turn initiate activation of the antioxidant system and thus increase the overall resistance of the body to other stress factors. Examples of this action include transient cooling by immersion into cold water or air at ultra-low temperature or liquid nitrogen vapor in a cryosauna. Further studies of the mechanisms of cold adaptation at both the organism and molecular levels provide not only a deeper understanding of the physiological reactions of humans and animals to oxidative stress, but also the opportunity to use this knowledge to develop effective preconditioning systems for the prophylaxis of diseases due to failure of metabolic rearrangements in response to different types of stress.

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