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Actions and effects of malignant tumor growth and chronic neurogenic pain exerted on the glutathione system in cardiac mitochondria in experimental animals

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Aim

The aim hereof is to study the glutathione system peculiarities in the cardiac mitochondria in experimental animals against the background of a tumor process, chronic pain and the combined effect both of chronic pain and the tumor process.

Materials and methods

The experiment has been carried out in female mice of the C57BL / 6 line (n = 28), aged 8 weeks, with an initial body mass of 21-22 g. The animals have been divided into the following groups: an intact group as the reference (n = 7), a test group with a reproduction of the chronic pain model (n = 7), a comparison test group (B16/F10), which covered the animals with standard subcutaneously inoculated melanoma B16/F10 (n = 7), and the main experimental group (chronic pain + B16/F10) of mice with B16/F10 melanoma inoculated 3 weeks after the chronic pain model development (n = 7). All rodents have been decapitated with a guillotine upon completion of 3 weeks of the experiment (on 21 day of the experiment). After decapitation, the animal hearts have been quickly removed with the use of coolants, and mitochondria have been isolated. In the obtained mitochondrial samples, using standard IFA test systems, the following levels have been determined: reduced glutathione (GSH) in nmol/L , oxidized glutathione (GSSG) in nmol/L, glutathione peroxidase-1 (GPx-1) in ng/mL, glutathione peroxidase-4 (GPx-4) in ng/mL, glutathione reductase (GR) in ng/mL, glutathione -S- transferase (GST) in ng/mL and superoxide dismutase-2 (SOD-2) in pg/mL. The statistics data have been calculated using the Statistica 6.0 software.

Results

In case of a chronic pain and a tumor process, in the cardiac mitochondria a low concentration of GPx-1, SOD-2 and a high level of GSSG are detected. The amount of GR increases in case of chronic pain and decreases under a malignant process. The combined effect of a chronic pain and a malignant process leads to a decrease in GSH against the background of an elevated GSSG level. The enzymatic component of the glutathione system (GPx-1, GR, GST) in the cardiac mitochondria in case of the combined actions and effects is characterized by a cumulative potential. The GSH/GSSG ratio in all experimental groups is statistically significantly lower than the intact values.

Conclusion

According to the results from this study, we may conclude that chronic pain and a tumor process exert systemically an effect on the animal's organism, but in doing so they affect different members of the regulation in the LPO-AOP system. A disorder in the glutathione system performance triggers an activation of molecular oxygen and a change in the redox potential that in its turn affects the subcellular level of the regulation and, in general, the entire cellular metabolism. The revealed abnormalities in the energy system of the cardiac mitochondria can be considered as stressor disorders. The combined effect of a chronic pain and a malignant process is treated by the organism as a double stress with an imbalance in the antioxidant glutathione system.

Keywords

Cardiac mitochondria, Experimental melanoma B_{16}/F_{10}, Chronic neurogenic pain, Mice, Glutathione system

Imprint

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Introduction

A tumor is a local process, but its development cannot but affect the activity of oxidative processes in other organs and tissues in the organism. A close connection between cardiovascular disorders and a tumor disease is a well-known fact. The spectrum of cardiac abnormalities in this case is quite wide and is associated with reactive free radicals, which damage not only the membranes of cardiomyocytes, but also their subcellular structures. It is common knowledge that the main "respiratory" organelle in the cell is a mitochondrion, which contains a large number of active enzymes and coenzymes in the respiratory chain and is a potential source of free radicals in a one-electron oxygen reduction [1, 2]. In order to control the level of active forms of oxygen (AFO), the mitochondria contain a multi-line defense system and mechanisms of antioxidant enzymes that allows maintaining the normal performance of the organelle without significant disorders. This system includes superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) [3, 2]. In ischemic states, a malfunctioning of the mitochondrial respiratory chain is observed with an increased free electrons flux and the formation of reactive oxygen species [4]. The active forms of oxygen themselves can modify proteins and nucleic acids that initiates lipid peroxidation, which leads to disintegration of the cell plasma membranes and as a final result to cell death [5]. In antioxidant protection, the most important role is assigned to the glutathione system [6]. Glutathione is found in all cells and tissues of living organisms and is of great importance for oxidation-reduction reactions due to the ability of the cysteine sulfhydryl group (SH) to reversibly enter into chemical combinations. Glutathione undertakes the following very important functions in the organism: it provides the functional activity of proteins, including enzymes, protects cells from active oxygen species, maintains the membrane activity and participates in the exchange of eicosanoids; it is a cysteine reserve; it makes an effect on the synthesis of nucleic acids, participates in the metabolism of xenobiotics, increases resistance to harmful factors and influences proliferative processes [7]. The glutathione antiperoxide system effectively protects cells from peroxidative stress, and usually serious damage may be observed only if it is deficient or depleted. Glutathione is the basic component of the cell redox buffer. It is believed that the main physiological role of reduced glutathione is to protect and restore thiol groups of protein molecules during their oxidation or binding. In addition to the cofactor supply, by this means the thiol redox control of enzyme activity is realized. In general, the glutathione system is crucial in resistance to various chemical factors of the environment, and it is an important wide-range protective mechanism of the cell [6].

Pathological processes in the organism are often accompanied by a pain syndrome. At present, the commonly accepted opinion is that chronic pain is not a symptom of any disease, but represents an independent disease [8]. It has been demonstrated that painful effects of different nature cause changes in the main types of metabolism, mobilization of adaptive metabolic mechanisms, tissue damage and, that is especially important, dysfunction of the vascular system [9]. Pain is one of the most common symptoms in cancer patients, and with the progression of the disease its occurrence rate increases. The type of pain and its intensity depend on the location of the tumor and the stage of the disease. Pain is present in 30 to 50% of the patients with antitumor therapy and in 65 to 90% of the patients showing oncological progression; 33% of the patients suffer from pain after the completion of an anti-cancer treatment [10]. As a rule, the causes of pain in cancer patients are multi-factorial direct and indirect effects of cancer, side effects of antitumor therapy and concomitant diseases. The pathophysiological mechanisms are combined, and they include both nociceptive (somatic and/or visceral) and neuropathic (neurological) components [10, 11]. In this regard, the study of the antioxidant processes under pathology accompanied by pain syndrome is the topical issue in current research.

The aim hereof is to study of the glutathione system peculiarities in the cardiac mitochondria in experimental animals against the background of a tumor process, chronic pain and the combined effect made by chronic pain and the tumor process.

Materials and methods

The study has been carried out in female mice C57BL /6 (n = 28), aged 8 weeks, with an initial body weight of 21-22 g. The animals have been provided by the Federal State Public Institution, the Andreevka Branch at the Research Center for Biomedical Technologies, the Federal Medical & Biological Agency (Moscow Region, Russia). The cell line of the mouse

B_{16}/F_{10} melanoma metastasizing into the lungs has been used herein. The tumor strain has been supplied by the Russian National Oncology Research Center "N.N. Blokhin" at the RAMS (Moscow, Russia).

Animals have been kept under natural lighting conditions with their free access to water and food. All the studies have been carried out in accordance with the requirements and conditions of the "International Guiding Principles for Biomedical Research Involving Animals" and the associated National Order Regulation No.267 dd. 19.06.03 "Approved Rules and Regulations for Laboratory Practices" issued June 19, 2003 by the Ministry of Healthcare of Russian Federation.

The rodents have been divided into the following experimental groups: an intact animal group as the reference (n = 7), a test group with a reproduction of the chronic model pain [12](n = 7), a comparison test group (B16/F10), which contains mice with standard subcutaneous transplantation of melanoma B16/F10 (n = 7), and the main test group (chronic pain + B16/F10) with the mice with the B16/F10 melanoma inoculated 3 weeks after the chronic pain model development (n = 7). The main test group mice (chronic pain + B_{16}/F_{10}) have been subjected to sciatic nerve ligation on 2 sides under Xylo-Zoletil anesthesia. Three weeks after healing the operation wound, 0.5 ml of a suspension containing the B_{16}/F_{10} melanoma tumor cells in a physiological saline at a 1:10 dilution has been injected subcutaneously under the right scapula. In the comparison test group (B_{16}/F_{10}), melanoma B_{16}/F_{10} has been subcutaneously inoculated at the same dose and in the same volume as it is the case with the main test group, but without reproducing the chronic pain model. With respect to the standard inoculation, the tumor appears in 100% of the cases, grows rapidly enough and on the 12-16th day of the growth metastasizes mainly hematogenously into the lungs (60-90%) and more rarely into the liver and the spleen. All manipulations with animals have been made in the special box. Tools, utensils and hands have been disinfected in a conventional manner.

All animals have been decapitated with a guillotine upon completion of 3 weeks of the experiment (on 21 day of the experiment). After decapitation the animal hearts have been quickly removed with the use of coolants, and mitochondria have been isolated according to the Egorov M.V. - Afanasiev S.A. method [13]. In the obtained mitochondrial samples, using the standard IFA test systems, determined have been the following data: reduced glutathione (GSH) in nmol/L (Bio Source, USA), oxidized glutathione (GSSG) in nmol/L (Bio Source, USA), glutathione peroxidase-1 (GPx-1) in ng/mL (Ab Frontier, South Korea), glutathione peroxidase-4 (GPx-4) in ng/mL (Clod-Clon Corporation, CNDR), glutathione reductase (GR) in ng/mL (Cusabio, CNDR), glutathione -S-transferase (GST) in ng/mL (Ivvundiagnostik, FRG) and superoxide dismutase-2 (SOD-2) in pg/mL (Ab Frontier, South Korea).

A statistical analysis of the results has been carried out using the Statistica 6.0 software (Stat-Soft, 2001). The data in Tables herein are represented in the form of M±m, where M is an arithmetic mean value, and m is the standard error of the mean. Variances have been assessed by the Student's criterion, and they have been considered to be statistically significant at $p < 0.05$.

Results

In the present study, it has been found that the level of oxidized glutathione (GSSG) in the cardiac mitochondria in animals (females) with chronic pain (test group) exceeds intact animal values by 1.6 times; at the same time, on the contrary, we have observed a decrease in the GR concentration by 36% and a reduction in the Gpx-1 and SOD-2 levels by 1.4 and 1.6 times, respectively (see Table 1 herein).

The concentration of reduced glutathione and GST in the cardiac mitochondria in the test group animals has been identified to be at the level of the intact values. In animals experiencing chronic pain, a deficiency in GPx-1 in the cardiac mitochondria is detected. Some researchers reveal a close relationship between a decrease in the GPx-1 concentration and a rise in the occurrence rate of cardiovascular disorders [14, 15, 16]. We have found that chronic pain in animals has not affected the level of reduced glutathione (GSH). It occurs probably due to the fact that the reduced glutathione is depleted from mitochondria much later than that from the cytosol [15]. GSH is synthesized in the cytosol only, from where it penetrates into the other compartments of cells. GSH penetrates through the inner membrane (Mi) into the mitochondrial matrix, where it reaches 10-15% of the cellular level and undertakes protection of the mitochondria from oxidative stress. Immediately in the cardiac muscle, the reduced glutathione penetrates through the inner membrane through the oxoglutarate carrier, which is

Table 1. Actions and effects of pathological processes on glutathione system in cardiac mitochondria in mice

Markers	Intact animals as the reference	Test group animals (chronic pain only)	Comparison test group (tumor B_{16}/F_{10} only)	Main experimental group (tumor B_{16}/F_{10} + chronic pain)
Reduced glutathione (GSH) (nmol/L)	170373,2± 14001	169562,9± 11722	213090,1± 16800[1,2]	113355,4± 91631,2,3
Glutathione oxidized (GSSG) (nmol/L)	361,16±28,2	590,79±49,8[1]	569,19±50,2[1]	474,16±41,3[1]
GPx-1 (ng/mL)	0,501±0,03	0,355±0,02[1]	0,31±0,02[1]	1,037±0,08[1,2,3]
GR (ng/mL)	1,591±0,08	2,159±0,1[1]	0,574±0,04[1,2]	9,324±0,7[1,2,3]
Glutathione-S-transferaseng/mL	7,254±0,5	7,928±0,6	7,165±0,5	8,886±0,6[1,3]
SOD-2(pg/mL)	1085,6±89,1	673,87±57,5[1]	525,1±49,8[1]	1099,6±87,7[2,3]

Notes: 1 – statistically significant in relation to the intact group values; 2 – statistically significant in relation to the test group values (chronic pain); 3 – statistically significant in relation to the comparison test group values (tumor B_{16}/F_{10}).

part of a metabolic pathway responsible for the electrically neutral GSH exchange.

In case of a malignant process, in the comparison test group animals detected has been an elevated level of GSH, which is 25% higher than it is the case with the intact group, and an increased level of GSSG, which is recorded to be 1.58 times greater if to compare with the intact animals. At the same time, the concentration of enzymes GPx-1, GR and SOD in the cardiac mitochondria has been reduced by 1.6, 2.8 and 2 times, respectively. If to compare with the values in the test group animals (chronic pain), statistically significant changes have been detected in the GSH and GR levels. Thus, the GSH level has been revealed 25.6% higher, while GR, on the contrary, has been recorded to be significantly lower, by 3.8 times. The concentration of glutathione -S- transferase (GST) in the cardiac mitochondria in the chronic pain and tumor-bearing animals has not demonstrated statistically significant differences from the intact group values.

The combined effect of chronic pain and the tumor process has contributed to a significant, 1.5 times, decrease in the level of GSH in the cardiac mitochondria, and a 1.3 times increase in GSSH, a 2.1 fold build up in GPx-1, a 5.8 fold increase in GR and a rise of 22,5% in GST in relation to the values in the intact animals. SOD has been recorded to be at the level of the intact values. If to compare with the values in the animals experiencing chronic pain only, the level of GSH has been reduced by a factor of 1.5, while the levels of GPx-1, GR and SOD have demonstrated a 2.9-, 4.3- and 1.6-fold increase, respectively. Comparing the result of the combined effect of chronic pain plus the tumor growth with the respective data in the group having tumor growing only, we have detected a 1.9 times lower GSH concentration, but the level of GPx-1 has been identified to be 2.2 times higher, the GR concentration has been found to be 16.2 higher, the GST level has increased by 24%, and a 2.1 times increase of the SOD concentration has been reported, too.

Table 2 given herein demonstrates the values of the glutathione system component ratios, reflecting the maintenance of the redox homeostasis.

Such an imbalance has produced a disorder in the performance of the physiological cascades of antioxidant enzymes. As an indicator of the antioxidant enzymes protective efficacy, the ratio of the SOD /GPx activity, which characterizes the systemic interrelation in the action of antioxidants and reflects a change in the enzymatic antioxidant protection balancing, can be used. Thus, noticed has been a decrease in the SOD/GPx-1 ratio under the tumor growth by 1.3 times and under the tumor growth & chronic pain combined action by 2 times in relation to the intact group of animals (see Table 2 herein). It should be noted that the SOD/GPx-1 ratio value in case of the combined action has been reported to be lower, by 1.7 and 1.5 times, respectively if to compare with the chronic pain and the tumor growth conditions. The GSH/GPx-1 ratio, as well as the SOD/GPx-1 ratio value, in case of the combined action, has been considered as the lowest as compared with the ratio values in the other study groups. Thus, the SOD/GPx-1 ratio has been reduced by 3.1 times, as compared with the intact values, by 4.4 times in the chronic pain group and by 6.3 times in the tumor growth group animals, respectively. The

Table 2. Indicators of the glutathione cascade in mice cardiac mitochondria against the background of pathological processes

Indicators	Intact animals as the reference	Test group animals (chronic pain)	Comparison test group (tumor B_{16}/F_{10})	Main experimental group (tumor B_{16}/F_{10} + chronic pain)
GSH/ GSSG	4,7±0,3	2,9±0,19[1]	3,7±0,28[1,2]	2,4±0,18[1,3]
GSH/GPx-1	3,4±0,27	4,8±0,3[1]	6,9±0,5[1,2]	1,1±0,08[1,2,3]
SOD/GPx-1	2,2±0,16	1,9±0,11	1,7±0,11[1]	1,1±0,06[1,2,3]
GR/GPx-1	3,2±0,21	6,1±0,47[1]	1,8±0,11[1,2]	9,0±0,7[1,2,3]

Notes: 1 - statistically significant in relation to the intact group values; 2 - statistically significant in relation to the test group values (chronic pain only); 3 - statistically significant in relation to the comparison test group values (tumor B_{16}/F_{10} only).

decrease in the GSH/GPx-1 ratio is associated with a change in the detoxifying properties of GPx-1 by switching between the elimination of hydroperoxides and the oxidation of GSH, in case of chronic pain and the tumor process, the consumption of GPx-1 increases, and an increase in the SOD/ GPx-1 ratio indicates a low production of hydroperoxides. The GR/GPx-1 ratio in the animals experiencing the pain syndrome has been recorded 2 times higher than the respective intact animal values, while under the tumor process, on the contrary, a 1.7-fold decrease has been detected. Besides, GR/GPx-1 in animals with the tumor process has been found 3.3 times lower in comparison with the chronic pain animal group values. The combined action (the tumor process plus chronic pain) has led to a significant increase in the GR/GPx-1 ratio as compared with the values in all study groups: by 2.8 times in comparison with the intact animal group, by 1.5 times if compared with the chronic pain animal group and by 4.8 times as against the comparison test group (tumor B16/F10). The GSH/GSSG ratio, reflecting the oxidation-reduction balance of glutathione, for the chronic pain animals in their cardiac mitochondria, has been recorded to be 1.6 times lower than the intact animal value, and 1.3 times lower than that reported under the tumor process condition. By comparing the values of the GSH/GSSG ratios for two mono-exposure cases (chronic pain, tumor growth), we have detected their higher values, a 1.3 fold increase therein, just under the growing tumor conditions. The combined action has resulted in a reduction of GSH/GSSG ratio in comparison with intact animals by 2 times and with the tumor bearing ones by 1.6 times, respectively (see Table 2 herein). The GSH/GSSG reduction indicates a decline in the glutathione system redox potential. With a decrease in the GSH/GSSG ratio under the oxidative stress conditions, thiol groups of proteins can be reversibly modified and form mixed disulfides between the SH groups of proteins and the SH groups of molecules with low molecular weight [17]. In this case, GSH serves as a "trap" for free radicals [2], and pathological states, including development of a neoplasm, results in a depletion of the GSH resource [18, 19].

Thus, any of the above described actions has provoked changes in the glutathione system performance in the cardiac mitochondria in the experimental animals. So, it has been established that the chronic pain syndrome induces an increase in oxidized glutathione and glutathione reductase, which is treated to be the only enzyme, which reduces oxidized glutathione, while the detected decrease in GPx-1 may indicate the consumption of selenium (Se) used to neutralize intracellular hydrogen peroxide. The review by A.I. Pashova et al. also provides evidence for a decrease in GPx in the organism under cardiovascular diseases and cancer [20]. Consequently, the chronic pain syndrome causes disorders and abnormalities in the cardiac performance.

A three-week tumor growth of melanoma is the terminal stage of development of the malignant process in experimental animals. At this stage of growth, melanoma reaches its maximum, often necrotizing and metastasizing. The animal's weight is found considerably reduced, and the organs lose their weight and acquire an atypical color [11]. In case of the tumor growth in animals, on day 21 of the experiment, the glutathione peroxidase and glutathione reductase parts of the antioxidant defense are inhibited in the mitochondria of the cardiac muscle, and, at the same time, GST turns out to be more resistant to the actions and effects of the tumor process. The level of oxidized glutathione is rather high, that indicates a shift in

the oxidative homeostasis towards peroxidation, but however a high level of reduced glutathione is also detected. It may indicate that, in case of the melanoma growth and development, resetting and reformatting of the performance of the organs takes place to satisfy the needs of the tumor itself. The peculiarities of the growth and development of melanoma, arisen due to its localization just on the skin, under the involvement of the entire skin cover vascular system, undoubtedly, affect the cardiac performance by resetting the mitochondrial energy system. We believe that from the cytosol, due to increased permeability of the intracellular membranes, a more intensive supply of reduced glutathione into the mitochondria is provided.

The combined action (tumor growth + chronic pain) on day 21 of the experiment has resulted in a stimulation practically of the entire glutathione enzyme system (GPx-1, GR, GST) and led to a depletion of reduced glutathione, while the accumulation of oxidized glutathione has been recorded to remain at the level produced by the mono-exposures, namely, under the conditions of the tumor growth or chronic pain. The combination of chronic pain and tumor growth has led to a general disproportion in the glutathione system components in the cardiac mitochondria. It is assumed that the transformed cells show an elevated level of antioxidant enzymes to protect cells from death [21, 22, 23]. In particular, it is known that GPx-1 is responsible for the regulation of cellular hydroperoxides and can protect from oxidative stress, but an excess of GPx-1 can also produce harmful actions and effects due to the lack of significant cellular oxidants [24, 25] that leads to restorative stress and is characterized by the absence of oxidants and / or reduction of excess equivalents [26]. Although the reductive stress may seem like a new concept: it has been known for some time that the absence of cellular oxidants can reduce the cell growth reactions. There is fresh evidence for additional cellular and physiological effects made due the absence of cellular oxidants and the accumulation of excess of reducing equivalents, including alterations in the formation of the disulfide bond of the protein, the diminished mitochondrial function and declined cellular metabolism. It is a matter of general experience that until the present time there has been no complete understanding of the physiological states capable of initiating a reductive stress. Such states as hypoxia, hyperglycemia and other stresses inhibiting the mitochondrial function cause an excessive accumulation of cellular reducing equivalents [27, 28, 29]. Besides, in some models of experimental cardiomyopathy, excessive reducing equivalents and an excess of GPx-1 should be attributed to the mechanism of cardiac dysfunction [33, 31]. In human individuals, GPx-1 is involved in the modulation of certain cancers and the occurrence of cardiovascular diseases [14]. Glutathione transferases conjugate to GSH some toxic products of LPO (nonenals, decinals), contributing to their excretion from the organism [20]. The main function of glutathione reductase is to reduce the oxidized glutathione to the sulfhydryl form of GSH, and in doing so to increase the level of reduced glutathione without enhancing its synthesis [32, 33]. We think that the revealed decrease in GSH accompanied by the increase in GST are most likely associated with the conjugation of reduced glutathione to toxins, caused by the combined action of the tumor growth and chronic pain syndrome.

Conclusions

According to the results of this study, we can conclude that chronic pain and tumor process have their actions and effects on the animal's organism in a systemic manner, but at the same time they address different components of the regulation in the LPO-AOP system. A disorder in the glutathione system performance initiates an activation of molecular oxygen and a change in the redox potential, which in its turn affects the subcellular level of the regulation and, in general, all cellular metabolism. The revealed disorders and abnormalities in the energy system of the cardiac mitochondria can be considered as stressors. The combined effect of chronic pain and malignant process is treated by the organism as a double stress with an imbalance of the antioxidant glutathione system.

Statement on ethical issues

Research involving people and/or animals is in full compliance with current national and international ethical standards.

Conflict of interest

None declared.

Author contributions

All the authors read the ICMJE criteria for authorship and approved the final manuscript.

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