cAMP concentrations in cardiac mitochondria and serum in the C57BL/6 mice under independent melanoma B16/F10 growth versus melanoma B16/F10 growth linked to chronic neurogenic pain


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Abstract

The aim of this research work is to study the cAMP level in the cardiac mitochondria and serum in the C57BL/6 strain mice of both genders under the independent melanoma B16/F10 growth versus the melanoma B16/F10 growth linked to chronic neurogenic pain (CNP).

Materials and methods. Mice of strain C57BL/6 (n=336) have been grouped as follows: the intact group of the mice (♂ n=21; ♀ n=21), the reference group (♂ n=21; ♀ n=21) with the reproduced CNP model, the comparison group (♂ n=63; ♀ n=63) to include the mice with melanoma B16/F10, and the main test group (♂ n=63; ♀ n=63) to cover the mice with the melanoma growth against the CNP background. Upon expiration of 1 week, 2 and 3 weeks of the melanoma growth, in the animals of the above experimental groups the cardiac mitochondria have been isolated with the centrifugation using high-performance refrigerated centrifuge Avanti J-E, BECMAN COULTER, USA. With ELISA Kit (RayBio USA) we have determined cAMP concentrations in serum and in the cardiac mitochondria.

Results. CNP has induced a decrease in the cAMP level in the cardiac mitochondria by a factor of 3.6 in the female mice only. In the animals of the comparison group the cAMP level in the heart has been increasing beginning with week 2 of the tumor growth on average by a factor of 4, while in the main test group starting from week 1 of the tumor growth it has been recorded 2-4 times higher and was depleted by the end of the experiment. As to the cAMP concentration in serum, the dynamics thereof has not been found to be in correlation with the cardiac mitochondrial data, and its concentration decrease has been recorded both in the females and the males.

Conclusion. So, the changes in the cAMP concentration in the cardiac mitochondria demonstrate their gender-specific feature; the female mice as against the males have responded to an independent impact produced by CNP. As to the main test group, CNP has stimulated an increase in the cAMP level in the cardiac mitochondria 1 week earlier than it is the case with the comparison group, and it has resulted in the full cAMP depletion by the 3rd week of the experiment.

Keywords

Melanoma B16/ F10, Mouse strain C57Bl/6, cAMP, Mitochondria, Heart, Chronic neurogenic pain

Imprint


Introduction

Acute myocardial infarction remains one of the most significant causes of death and post-event functional disability in population throughout the world [1]. In the circumstances the myocardium is deprived of blood that leads to an acute mass death of its cells and as a consequence results in the loss of cardiac contractility, collagen scar formation and subsequent progressing remodeling as well as heart insufficiency [2].

Cyclic adenosine monophosphate (cAMP) is a common second messenger, and it plays a decisive role in the intracellular signal transduction in response to external or internal stimuli. cAMP is synthesized from adenosine triphosphate (ATP), and its intracellular
level is regulated by the activity of adenylate cyclase, phosphodiesterases and A kinase anchoring proteins (AKAP) [3]. cAMP is responsible for regulation of different cell processes including proliferation, migration and differentiation of cells plus undertakes control over a wide range of other processes, which have a wide variety and which sometimes may act in opposite directions, that include, for instance, control of cardiac excitability and contractility, pain signal transduction, gene transcription, mitochondrial homeostasis and cell death [4, 5]. The wide range of the physiological responses initiated by various stimuli is realized via the cAMP-dependent pathways owing to the cAMP molecule compartmentalization in a cell, since a certain specific pool of protein kinases in different intracellular compartments is activated [6].

At present, there is no doubt that the cAMP-dependent pathways participate in realization of different physiological and pathological states, diabetes, cardiovascular diseases and malignant tumor growth among them [3, 4, 7].

cAMP is the major regulator of mitochondrial metabolism, but so far a precise mechanism of its action remains not clear [8]. Under the physiological conditions, the cAMP signal transduction plays a key role in the regulation of the cardiac function. Mitochondria are responsible for energy production for the cardiovascular performance, and the cAMP signal transduction is in close touch with the mechanism of their operation [9, 10]. The activation of the cAMP-dependent intracellular signal pathway represents an adaptation of cardiomyocytes to various extracellular stimuli and initiates an activation of some specific intracellular effectors, which provide the proper cell response. It follows that for a cell it is essential to maintain a strictly controllable transduction of the cAMP signals in space and time [11]. It is suggested that the signal cAMP pathways are cardio-protective under variable conditions [1].

cAMP activates two main pathways of the signal transduction in the heart: the cAMP-dependent protein kinase and guanine-exchange protein, activated directly by cAMP: Epac [12]. Epac being an important cAMP effector is a participant in different physiological mechanisms of cardiovascular diseases. Epac acts on different pathways including the regulation of the ion level, cardiac hypertrophy and fibrosis, cardiomyocyte apoptosis and angiogenesis [13]. The proper performance of mitochondria is vital in the maintenance of the physiological functions of the circulatory system. And vice versa a disorder in the regulation of the mitochondrial function is of crucial importance in CVD pathogenesis as well [9, 10].

Earlier, in our experiment, we have shown that the growth of melanoma against the background of chronic neurogenic pain leads to development of myocardial infarction in mice (Frantsiyants E.M. et al., 2021). Moreover we have revealed that comorbide pathology (diabetes mellitus, chronic neurogenic pain) linked to a malignant process dramatizes a dysfunction of the cardiac mitochondria with destabilization of the respiratory chain mediated by the processes of free-radical oxidation [14].

The aim of this research is to study the cAMP level in the cardiac mitochondria and serum in the C57BL/6 strain mice of both genders with melanoma B16/F10 under its growth without chronic neurogenic pain (CNP) and against the background thereof.

Our experimental study was conducted in the C57BL/6 strain mice (n=336), aged 8 weeks, with an initial individual body mass of 21-22 g. The experimental animals were supplied by the Federal State Medical & Biological Institution “Research Center of Biomedical Technologies at the Federal Medical & Biological Agency” (Branch Andreevka, Moscow region); and they were kept under natural lighting conditions with no restrictions on access to water and food. The study was conducted in accordance with the “International Recommendations on Pursuance of Medical & Biological Research in Animals” and Order No.267 “Approval of Regulations on Laboratory Practice” dd. 19.06.03 issued by the Ministry of Health Care of the Russian Federation. The produced Research Report was approved by the Bioethics Commission at the FSBI “The National Medical Research Center for Oncology”, the Ministry of Health of Russia, Ethics Committee Record No.2 dd. 31.05.2018. All manipulations with the animals were conducted in a box in accordance with all applicable rules and regulations of aseptics and antiseptics.

In our studies, we have used mice melanoma line B16/F10 delivered by the FSBI “National Medical Research Center for Oncology named after N.N.Blokhin” at the Ministry of Health of the Russian Federation. The melanoma line cells have been transplanted as a volume of 0.5 ml of a suspension containing the B16/F10 melanoma tumor cells in a physiological saline at a 1:10 dilution by subcutaneously injecting under the right scapula.
The model of chronic neurigenic pain (CNP) has been reproduced by sciatic nerve ligation on 2 sides under Xylo-Zoletil anesthesia [15].

The female and male animals have been randomly divided into experimental groups as follows: the intact group (♂n=21; ♀n=21), the reference group (♂n=21; ♀n=21) to reproduce CNP, the comparison group (♂n=63; ♀n=63) to cover mice upon standard subcutaneous transplantation of melanoma B16/F10, and the main test group (♂n=63; ♀n=63) to include the mice with the CNP model reproduction followed by the melanoma B16/F10 transplanted 3 weeks after CNP modeling.

The animals have been decapitated with a guillotine; the mice in the main test group and in the comparison group have been guillotined upon expiration of 1 week, 2 and 3 weeks of the experimental melanoma growth. After decapitation the animal hearts have been quickly harvested with the use of coolants, and mitochondria have been isolated with the centrifugation employing the high-performance refrigerated centrifugue Avanti J-E, BECMAN COULTER, USA. With ELISA Kit (RayBio USA) we have determined cAMP concentrations in serum and in the cardiac mitochondria.

Our statistics data obtained have been processed using the Statistica 10.0 software package. The Shapiro-Wilk criterion test has been applied to test the delivered data for fit to the normal distribution (for small sample sizes). The Mann-Whitney test (for independent samples) has been employed to evaluate the significance of variances between samples that has been taken to be p<0.05. Comparing two or more independent samples of equal or different sample sizes, we have applied the Kruskal-Wallis method, and in case of a posteriori comparisons we have utilized the Shapiro-Wilk test with correction of the significance level (p<0.017). 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.

The results obtained in assessing the cAMP levels in mitochondria isolated from the cardiac muscle in the mice of both genders are given in Table 1 herein.

We have revealed some gender-specific features of the cAMP concentration in the cardiac mitochondria in the intact animals as given below: in the females this index has been found 3 times higher than it is the case with the males. The impact made by CNP on the cAMP level in the cardiac mitochondria has also demonstrated its gender-specific aspect: the cAMP level in the males in the cardiac mitochondria has not shown any statistically significant differences from the data recorded in the intact mice, while in the females this parameter has shown a decrease by a factor of 3.6.

Upon expiration of one week of the independent melanoma B16/F10 growth, no changes in the cAMP level in the cardiac mitochondria in the animals of both genders have been reported, however upon completion of two weeks of the experiment we have detected an increase in the cAMP level in the males by a factor of 9.4 and in the females by a factor of 4.2, respectively, as against the data recorded in the intact mice. Upon expiration of 3 weeks we have found a decrease in the cAMP level in the cardiac mitochondria in the males by a factor of 2.5, but however it has remained still 3.8 times higher than it is the case with the intact animals, while in the females the cAMP concentration in the cardiac mitochondria has reduced by a factor of 4.6, and it has not shown any differences from the values recorded in the intact females.

<table>
<thead>
<tr>
<th>Group</th>
<th>cAMP (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>Intact animals</td>
<td>0.19±0.011</td>
</tr>
<tr>
<td>Reference group (CNP)</td>
<td>0.21±0.012</td>
</tr>
<tr>
<td>Comparison group</td>
<td></td>
</tr>
<tr>
<td>Week 1 B16/F10 growth</td>
<td>0.27±0.017</td>
</tr>
<tr>
<td>Week 2 B16/F10 growth</td>
<td>1.79±0.092</td>
</tr>
<tr>
<td>Week 3 B16/F10 growth</td>
<td>0.73±0.057</td>
</tr>
<tr>
<td>Main test group</td>
<td></td>
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<tr>
<td>Week 1 B16/F10 growth+CNP</td>
<td>0.87±0.058</td>
</tr>
<tr>
<td>Week 2 B16/F10 growth+CNP</td>
<td>1.14±0.059</td>
</tr>
<tr>
<td>Week 3 B16/F10 growth+CNP</td>
<td>0.02±0.001</td>
</tr>
</tbody>
</table>

Note: statistically significant differences: p1 – between the intact females and males; p2 – in relation to the level in the intact group; p3 – in relation to the level in the chronic neurogenic pain group (the reference); p4 – in relation to the level at the previous period of the experimental study.
Upon expiration of one week of the melanoma B16/F10 growth against the background of CNP, the cAMP concentration in the cardiac mitochondria in the males and females has increased by a factor of 4,1 and by a factor of 2,1, respectively, as compared with the data recorded in the animals in the reference group (with CNP). Upon completion of two weeks of the experiment we reported an increase in the cAMP concentration in the cardiac mitochondria in the above animals by a factor of 1,3 and by a factor of 4,7, respectively, as against the previous stage of the study, and it has been found 5,4 and 10 times higher than the respective data in the cardiac mitochondria in the males and females in the reference group. Three weeks later we have revealed a dramatic drop of the cAMP level in the cardiac mitochondria in the males and females with the melanoma growth against the background of CNP by a factor of 57 and by a factor of 26,5, respectively, as against the data recorded in the reference group.

It can be said that we have revealed a different dynamics in the cAMP concentration in the cardiac mitochondria in the mice of both genders under the melanoma growth without CNP and that against the CNP background. Under the normal conditions, we have recorded higher concentrations of cAMP in the cardiac mitochondria in the males and females with the melanoma growth against the background of CNP by a factor of 57 and by a factor of 26,5, respectively, as against the data recorded in the reference group.

It can be said that we have revealed a different dynamics in the cAMP concentration in the cardiac mitochondria in the mice of both genders under the melanoma growth without CNP and that against the CNP background. Under the normal conditions, we have recorded higher concentrations of cAMP in the cardiac mitochondria in the intact female mice as compared with the male mice, and the independent CNP has produced its impact on the cAMP level in the cardiac mitochondria in the females only by inducing the level lowering. Under the CNP-free growth of melanoma (in the comparison group), the change in the cAMP level in mitochondria in the animals of both genders has been detected in the period starting with week 2 of the experiment only. As to the main test group, under the melanoma growth against the CNP background, a rise in the cAMP level in the cardiac mitochondria in the males and females has been found at an early date: upon completion of week 1 as well as by week 3 of the experimental tumor growth we have found its depletion, which has been recorded to be more pronounced in the males.

Our next step in the experiment has covered investigations on cAMP concentrations in serum in the mice. The results from the investigations are summarized in Table 2 herein. In this case no statistically significant differences in the cAMP concentrations in serum in the animals depending on the gender and CNP impact have been revealed. Under the CNP-free melanoma growth conditions (in the comparison group), as against the respective data in the intact animals of the corresponding gender, we have recorded at all stages of the experiment lowering of the cAMP level in serum as follows: after 1 week of the experiment in the males and females by a factor of 1,8 and by a factor of 1,5, respectively; upon completion of 2 and 3 weeks of the experiment the data in question have remained practically at the above mentioned level.

### Table 2

**cAMP concentrations in serum in mice**

<table>
<thead>
<tr>
<th>Groups</th>
<th>cAMP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>Intact animals</td>
<td>149,6±6,393</td>
</tr>
<tr>
<td>Reference group (CNP)</td>
<td>126,2±7,554</td>
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<tr>
<td>Comparison group</td>
<td></td>
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<tr>
<td>Week 1 B16/F10 growth</td>
<td>82,3±5,367</td>
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<td>(p=0,0000)</td>
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<tr>
<td>Week 2 B16/F10 growth</td>
<td>110,5±7,146</td>
</tr>
<tr>
<td></td>
<td>(p=0,0015)</td>
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<tr>
<td>Week 3 B16/F10 growth</td>
<td>80,0±5,278</td>
</tr>
<tr>
<td></td>
<td>(p=0,0000)</td>
</tr>
<tr>
<td>Main test group</td>
<td></td>
</tr>
<tr>
<td>Week 1 B16/F10 growth+CNP</td>
<td>132,2±7,934</td>
</tr>
<tr>
<td></td>
<td>(p=0,0000)</td>
</tr>
<tr>
<td>Week 2 B16/F10 growth+CNP</td>
<td>70,4±5,944</td>
</tr>
<tr>
<td></td>
<td>(p=0,0000)</td>
</tr>
<tr>
<td>Week 3 B16/F10 growth+CNP</td>
<td>144,2±9,383</td>
</tr>
</tbody>
</table>

Note: statistically significant differences: \(p_1\) – between the intact females and males; \(p_2\) – in relation to the level in the intact group; \(p_3\) – in relation to the level in the chronic neurogenic pain group (the reference); \(p_4\) – in relation to the level at the previous period of the experimental study.

A somewhat different dynamics in the serum-related data has been detected in the mice with the melanoma growth against the CNP background. So, upon completion of week 1 of the experiment, in the males no statistically significant decrease in the cAMP level in serum has been recorded as compared with the data in the animals with CNP, while in the females a decrease therein by a factor of 1,8 has been reported. Two weeks later, in the males the cAMP level in serum has been found 1,8 times lower, while that found in the females has shown no significant differences from the values recorded in serum in the females with CNP. 3 weeks after the inoculation with the tumor cells the cAMP level in serum in the male and female mice under the melanoma growth against the CNP background has demonstrated no significant differences.
from the values reported in the CNP mice of the respective gender.

It should be noted that the dynamics of the serum cAMP concentration data has not been found in correlation with the changes observed in the data pertaining to the cardiac mitochondria data and is most likely specific to the tumor process as a whole, with reducing energy capabilities of the organism, that might be attributed to a correction impact produced by CNP as comorbidity.

Discussion

It is known that cAMP undertakes the function of a secondary intracellular messenger-mediator in response to actions of the primary messengers, which have a short period of degradation: for example, of some hormones and neurotransmitters. The signal transduction of cyclic adenosine monophosphate protein kinase A, i.e. cAMP-PKA signaling, plays a key role in neuropathic and inflammatory pain [17]. The main objective of cAMP is to activate the cAMP-dependent ferment family, the members of which are given the name protein kinase A. The cAMP signal is a participant in the mechanism of the mitochondrion-dependent apoptosis, and it is of the essence in the signal transduction inside the cell. The signaling pathway of cAMP is also decisive in modulating an apoptotic response to different stimuli, and changes in the level of the cell cAMP may induce or suppress apoptosis depending on the cell type [18].

In our investigations we have revealed the gender-specific features in the cAMP level changes in the cardiac mitochondria as a response to CNP, in particular the cAMP level decrease found in the female mice only. It is known that the gender makes its own influence on the heart, and the initial performance of the heart in women is found more stable in pre-menopause so that the females demonstrate a lower CVD risk as compared with men of the same age cohort [19]. In our research work we have detected that the cAMP level in the cardiac mitochondria in the intact female mice is higher than that recorded in the males.

We have shown that the cAMP level in the cardiac mitochondria in response to CNP has decreased in the female mice only, while in the male mice no changes therein have been detected. It is common knowledge that estrogens are capable of activating the cAMP-dependent pathways and increase the cardiac contractility under the normal conditions [20]. However responding to a rising demand (for instance, under exercises, stress and diseases including CNP) the female hearts and myocytes are contracting weaker than it is the case with the hearts in the male cohort. Possibly it may be attributed to the fact that estrogens counteract the function of catecholamines [21] and, in doing so, reduce the cAMP levels inside the cells and suppress the Ca 2+ release [22].

Probably, the recorded increase in the cAMP level of the cardiac mitochondria in the group of the animals with the melanoma B15 growth only, starting with week 2 of the experiment, can be treated as a manifestation of tumor stress. Experimental studies on animals demonstrate that stress may play a role of a potential factor in the cAMP and protein kinase S regulation [23]. Whereas tumor stress in the group of the mice with the independent melanoma growth may be classified as an acute one, in the group with the combined process we deal with acute stress due to the melanoma inoculation, having developed earlier against the background of chronic stress produced by CNP. We are inclined to believe that the obtained evidence data on the increase in the cAMP level in the cardiac mitochondria under the independently growing melanoma, beginning with week two of the experiment, and in the cardiac mitochondria in the mice under the melanoma growth against the background of CNP during week 1 and 2 of the experiment should be treated as a compensatory effect aimed to avoid the death of mitochondria and naturally cardiomyocytes. So, it is known that the high level of cAMP in the myocardium first aids in the performance of the heart exposed to stress, but however the chronically remaining high level thereof promotes unfavorable remodeling of the heart, contributes to the death of myocytes, replacement fibrosis and progressing degradation of the heart function (i.e. a vicious cycle of heart failure) [11].

In our previous experimental work we have demonstrated that progression of melanoma against the background of CNP leads to myocardial infarction in mice and is accompanied by hemorrhage, necrosis foci, ruptures of some individual cells, focal infiltration of leukocytes, fibrinoid necrosis of vessel walls and cardiac chamber enlargement. This sort of alterations of the cardiac muscle has not been found in macroscopic examination and morphological study of the cardiac wall in case of the independent growth of the tumor [24].

We assume that a sudden drop of the cAMP level in the cardiac mitochondria in the animals in the main test group upon completion of 3 weeks of the experi-
ment may be connected with ischemia. It is interestingly that ischemic preconditioning initially increases the cAMP level in an intermittent ischemia (i.e. in the phase of preconditioning), but however later it reduces the cAMP accumulation in a continuous long time ischemia (i.e. in the main phase of ischemia) possibly due to the desensitization of β-adrenoreceptors or the compensatory activation of phosphodiesterase [25].

Earlier we have demonstrated that under the standard growth of melanoma, just starting from week two after the tumor transplantation, we have noted a decrease in the AIF level in the cardiac mitochondria in the female mice as a smooth transition to the hypoxic type of respiration and the production of ATP [23], from which cAMP is derived.

In addition, the AIF deficiency leads to a higher sensitivity by mitochondria to oxidative stress as a damaging factor [26]. It seems likely that the increase in the cAMP concentration in the mice with the independent growth of melanoma B16/F10 only exactly since week two of the tumor growth is caused by this occasion. Under the tumor growth conditions against the CNP background there is disjointing of the coupled processes of the respiration, phosphorylation and the cAMP response: beginning with week 1 after the tumor inoculation, the AIF concentration in the cardiac mitochondria in the mice rises sharply, but however in this case we deal with an increase of the cAMP level, too.

It is evident that the changes in the cAMP levels in different directions, as recorded upon completion of 3 weeks of the experiment, bear witness to the fact that there are weakening of the compensatory function, which however remains sufficient to protect the cardiac mitochondria from damage in the group of the animals with the independent growth of melanoma, and that there is a an adaptation collapse and the appearance of apoptotic and/or necrobiotic damage in the group with combined pathology.

In this experiment, the cAMP concentration in serum in the mice cannot be connected with the metabolism processes in the cardiac muscle; since the B16 melanoma cells have constitutively the increased intracellular cAMP levels [27]. It is a common knowledge that an increase in the intracellular cAMP level activates synthesis of melanin, DNA reparation and survival pathways in melanocytes [28]. The cAMP pathway demonstrates its non-linear action that may be of synergetic or antagonistic type, depending on the signaling environment [29].

The cAMP level in serum is most likely to be a mirror of the metabolic processes occurring in the tumor cells or in the tumor-bearing organism in general, but this subject requires further investigations.

Hence the obtained evidence data confirm that the changes in the cAMP concentrations in the cardiac mitochondria are of pathological significance for developing cardiovascular disorders in the animals with the growing tumor, while the cAMP level in serum does not demonstrate the specificity and characterizes the general state in the tumor disease.

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

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

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