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On Novel Features of the Proton Leak and Possibility of Uncoupling Population of Mitochondria in Brown Adipose Tissue

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Abstract In many studies on succinate as a substrate, the respiration of brown adipose tissue mitochondria is partially uncoupled that is associated with proton leak. NAD-dependent substrates (pyruvate + malate) were also used in a number of works. Oxidation of NAD-dependent substrates occurs with greater coupling where the respiration rate is higher and state 4 (resting state of mitochondrial respiration) lower than that with succinate. It is suggested that the oxidation of these substrates occurs in different ways. Further, a series of studies with a large number of oxidation substrates (NAD-dependent substrates, succinate, NADH and ascorbate + cytochrome c) was performed. It was found that NADH and ascorbate oxidized in BAT mitochondria very intensive and completely uncoupled with ATP synthesis. To explain these data, it is necessary to carry in certain changes in the existing proton leak scheme. In BAT mitochondria, it was also found high activity of Mg^{2+}-stimulated ATPase, sensitive to olygomycin. It has also high activity than DNP-stimulated ATPase. To explain of oxidation of many substrates on uncoupling way and existence of Mg^{2+}-stimulated ATPase in BAT mitochondria the review suggests an alternative concept, according to which two mitochondrial subpopulations are present in the suspension of BAT mitochondria. One of them carries out synthesis of ATP as generally accepted, and the second one – with a highly porous membrane, oxidizes exogenous NADH (GDP sensitive), ascorbate (+cytochrome c) and other substrates uncoupled way. Apparently, a special population of mitochondria can exist in brown fat that can oxidize many substrates in uncoupling way for thermogenesis.

Keywords Brown adipose tissue mitochondria, Proton leak, Coupled and uncoupled respiration, Thermogenesis

1. Introduction

Thermogenesis is an important part of the metabolism of many tissues, including that of beige fat, but especially intensively in brown adipose tissue [1-3]. It may be the result of different substrates oxidation, and therefore it is of interest to consider the properties of their oxidation in mitochondria. As the history of mitochondrial research shows, their enzymatic components are interrelated rather labile and can easily change electrons and protons flow, and ATP synthesis efficiency. In particular, NAD-dependent substrates are characterized with high levels of coupling of oxidation and phosphorylation, which distinguishes them from succinate. Even the opinion was expressed that succinate and NAD-dependent substrates are oxidized in different ways [4]. In addition, it was shown that only in the oxidation of succinate, and not in pyruvate with malate, much more heat is produced in mitochondria in state 4 [5]. This fact also indicates a great difference in the pathways of succinate and other substrates utilization in mitochondria. These problems are discussed poorly in publications, although this difference may be an important point in relation to their participation in a proton leak. As mentioned above, succinate is often used in research this problem in BAT mitochondria that is oxidized with less coupling, which provides leak of protons and thermogenesis. Concerning NAD-dependent substrates, there is much information about their strong coupling with the ATP synthesis, but little is known about their participation in proton leak [4].

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2. Different Concepts of Thermogenesis in BAT Mitochondria

According to the existing conception [6, 8, 9], all BAT mitochondria are involved in proton leak, and their inner membrane contains the protein thermogenin (UCP1 – uncoupling protein). This protein causes an increase in the permeability of the inner mitochondrial membranes, causing

proton leak. This leads to uncoupling of oxidative phosphorylation in mitochondria, which should be approximately the same for different oxidation substrates. However, the results of the study do not always agree with this possibility, since on succinate oxidation mitochondria possess a higher proton leak [4, 7, 10].

It is very interesting to give one more example from the history of oxidation substrates study. In particular, in muscle and cardiac mitochondria, it was noted that simultaneously with low uncoupling of succinate oxidation, intensive oxidation of exogenous NADH occurs [10-14] without coupling with ATP synthesis. Consequently, these two substrates are related somehow.

In individual publications, it is shown that NADH is intensively oxidized in BAT mitochondria [15] that was left unattended by researchers. It can be assumed that uncoupled oxidation of succinate and NADH is connected through UCP1 that increases the mitochondrial membrane permeability. It can be generally considered as the NADH oxidation phenomenon as damage of mitochondrial membranes [18]. Perhaps this factor was the main cause of the weak attention of researchers to the uncoupled oxidation of NADH in mitochondria.

Previous studies have conducted a special research of this problem using isolated cardiomyocytes [16, 17]. In these cells, there is intensive oxidation of exogenous NADH after their treatment with digitonin, which enhanced at the addition of cytochrome c. This study allows a more confident and critical look at the statement of some researchers about the possibility of an artifact in the results. However, the data obtained above made possible the interconnection between the loosely-coupled oxidation of succinate and the completely uncoupled oxidation of exogenous NADH. The approach used showed that not only succinate, but also NADH, participates in the proton leak that significantly expands the capabilities of the proton leak system. Moreover, with this methodical approach it was possible to show that a special subpopulation of mitochondria function in the cells for the purposes of proton leak and tissue thermogenesis. Consequently, two types of mitochondria can exist in the studied suspension: one of them carries out ATP synthesis (coupled mitochondrial population), another type of mitochondria perform uncoupled respiration (proton leak). Loosely coupling of succinate oxidation can be explained by its oxidation in two populations of mitochondria, therefore coupled and uncoupled elements are noted. The uncoupled oxidation of added NADH can be explained by its oxidation only in uncoupled mitochondrial populations, since NADH does not penetrate the coupled mitochondria [18].

The membrane is a complex structure and its permeability in mitochondria is an important indicator of their functioning. In particular, many environmental conditions were compared in order to understand the mechanism of proton transport during proton leak in mitochondria [19]. Proton leak depends little on many factors, but it can strongly depend on the modification of mitochondrial membrane by the presence of uncoupled proteins [19].

In the literature on proton leak study, it is generally accepted that this process manifests itself as a partial uncoupling of phosphorylating mitochondria. Such a concept allows for the existence in the cell of one mitochondrial population, where simultaneously coupled and uncoupled respiration with ATP synthesis occur [9, 20]. Some schemes of this design were proposed, but there is no definitive opinion on this question. In the literature, great importance is attached to the activation of proton leak by fatty acids [2, 21-23]. It is known that fatty acids themselves are good substrates for oxidation in mitochondria, and then they are uncouplers of oxidative phosphorylation so they increase proton permeability in lipid membrane [21]. Under the influence of these effects, fatty acids could activate proton leak in the mitochondria even if it was not UPC1 in the membrane. Even without fatty acids addition, succinate is a good activator of proton leak in uncoupled mitochondria. A good activator of proton leak is also NADH that is intensely oxidized in uncoupled mitochondria that previously it was not used to study proton leak in BAT mitochondria and other tissues.

Membrane studies of thermogenin with fatty acids. The carried out researches on bilayer artificial membranes and liposomes show that not all the received data are coordinated with each other. Most studies have shown that fatty acids are required to activate proton conductivity, and the nucleotide - GDP suppresses this process [24-27]. However, not all fatty acids activate proton conductivity; even some of them suppress [25].

In the patch-clamp experiments [23], not all of the above conclusions are confirmed. So, there is no special mention of the necessity of fatty acids and GDP does not suppress the proton conductivity on the patch-clamp model that contradicts the generally accepted point of view.

Recently, using the patch-clamp method, the transport of fatty acids in the antiport with the proton has been demonstrated [23], that indicates the permeability of protons and fatty acids throw this membrane. Previously it is believed that their transport occurs along an simportic scheme [2] Therefore, this problem needs to be further studied taking into account the questions of proton and fatty acids symport.

According to opinion of some researches [28] activation of proton conductivity by fatty acids is considered a secondary issue, and channel conductivity is more important. The results obtained by the authors [2, 28] correspond to both the channel and transport models of proton transfer through the membrane.

The symport variant of fatty acids exchange to proton through the inner mitochondrial membrane [2] corresponds more to the idea of high permeability of the inner mitochondrial membrane to various substances. However, the lack of experimental data does not allow a broader discussion of the results of these interesting studies.

3. Modification of Artificial Membranes Permeability by Thermogenin

One more modification of proton transport can be considered, when UPC1 (thermogenin) forms a conduction channel in the artificial bilipid membrane (BLM). According to available data [29] conductivity of the formed polypeptide channel is 4.5 nSm in 1 M of KC1, and the estimated diameter of this channel is about 20 Å. It can be assumed that such channels are formed in the inner mitochondrial membrane and completely permeable to different substrates of the Krebs cycle, NAD and NADH, as well as proteins with low molecular mass. It should also be noted that porin could have a certain attitude towards thermogenin, so formed conduction channels in BLM are close in both proteins [30].

In this work [29] thermogenin was isolated by the method used to isolate porin from the outer membrane. Thermogenin was isolated from inner mitochondrial membranes of BAT of newborn guinea pigs.

This methodical modification turned out to be an important point, since it allowed us to obtain a thermogenic channel with high permeability in BLM [29]. Typically, this technique is used to obtain porin from the outer mitochondrial membrane [30].

It was reconstructed in BLM for proton conductivity research. Needless to say, the effects of fatty acids have not been tested in experiments, since the membrane conductivity is high without it. In addition, a specific inhibitor of GDP has not been used, that is a specific inhibitor of proton leak in isolated mitochondria. But channel formation is not known whether this channel is selective for proton. It is more likely that many compounds can pass through this channel through the specified size. Also, the content of porin in the isolated preparation is known, since thermogenin and porin have similar molecular weights [30].

Mitochondria with such pores [29] cannot synthesize ATP and they may not have a membrane potential. Moreover, these mitochondria with such pores may have an uncoupled respiratory chain and ATPase. At the same time, these mitochondria are well involved in proton leak, oxidizing different substrates by uncoupling way [10, 16, 17]. Such a proton leak model was previously considered [10].

Why succinate oxidation has a loose coupling? According to the existing concept the main feature of proton leak is the loose coupling of all mitochondria [9, 19, 20]. Succinate is oxidized in mitochondria with low coupling even with the addition of BSA, i.e. without the presence of fatty acids [10, 16]. In the analysis of proton leak, other substrates (NAD-dependent substrates) are weakly used for proton leak oxidation [10, 17].

The above-mentioned questions on uncoupling oxidation of succinate and NADH are discussed here, since oxidation of these substrates have not been fully studied in the analysis of proton leak. If all substrates were oxidized with loose coupling in mitochondria, there would be no reason to discuss the issue of proton leak. Why does asymmetric uncoupling oxidation of substrates occur in mitochondria? However, the unequal participation of succinate and NAD-dependent substrates in proton leak prompts new studies to understand the mechanisms of this difference. Earlier, a diagram explaining this difference was presented based on the study of proton leak in rat cardiac mitochondria [10, 16]. As described above [10], in the preparation of cardiac mitochondria succinate is oxidized in both types of mitochondria, therefore it has loose coupling. Succinate dehydrogenase is involved in succinate oxidation, the coenzyme of which is FAD, is directly linked to the respiratory chain. Therefore, succinate oxidation is not disturbed in uncoupled mitochondria. Oxidation of succinate oxidation is not disturbed even when the mitochondrial membranes are destroyed by freezing and thawing in contrast to the oxidation of NAD-dependent substrates [31]. Therefore, in uncoupled mitochondria, we cannot see the oxidation of NAD-dependent substrates until we add coenzyme NAD that is noted for cardiac mitochondria [10]. Dehydrogenases of NAD-dependent substrates depend on coenzyme NAD that is not attached to the respiratory chain and is water soluble. Therefore, this coenzyme outflows uncoupled mitochondria with high membrane permeability. This condition does not allow oxidation of NAD-dependent substrates in such mitochondria. However, the addition of NAD to the incubation medium promotes the oxidation of these substrates. Generated NADH from NAD-dependent substrates oxidizes easily in such mitochondria, and cytochrome c enhances its oxidation [10]. In tissue homogenates, NADH oxidation occurs with high intensity without cytochrome c [17].

In BAT mitochondria, the oxidation of pyruvate with malate also occurs intensively, where apparently there is enough endogenous NAD to accelerate the oxidation of NAD-dependent substrates [17].

It should be noted that under the suitable experimental conditions the oxidation of all these substrates is effectively suppressed by GDP [15] as the proton leak inhibitor [3]. These facts suggest that NADH oxidation in BAT mitochondria, like succinate, is blocked by GDP. Therefore, this oxidation is directly related to the proton leak system.

The two mitochondrial populations may have a definite relation to reverse electron transfer in mitochondria. This line of research has unclear positions since the time of Chance [32]. For their further study, we need new analytical approaches. The reverse transfer of electrons is clearly manifested in mitochondria at fluorimetric registration of pyridine nucleotides reduction. In the succinate oxidation, a significant excess of a reduction level of pyridine nucleotides can be seen than during oxidation of NAD-dependent substrates. Briefly, two mitochondrial subpopulations may participate in excess pyridine nucleotides reduction during succinate oxidation [32, 33]. Its oxidation may suppress NADH oxidation in two subpopulations of mitochondria that is followed by substantial increase of NADH without “reverse electron transport”. NAD-dependent substrates are oxidized only in one mitochondrial subpopulation therefore NADH is increased small pyridine nucleotides fluorescence

as commonly observed in experiments [32, 33]. Therefore, it is possible to explain alterations in pyridine nucleotides reduction in mitochondria even without reverse electron transfer during oxidation of succinate. This interesting phenomenon needs further analysis.

It is known that the inner membranes of BAT mitochondria contain uncoupling protein, thermogenin that isolated from BAT mitochondria long ago [6]. This protein occupies about 10% of the total amount of mitochondrial proteins and increases membrane permeability [6]. However, its specific mechanism is unclear. According to the available data [9] it can cause mitochondrial uncoupling by more than half (succinate as a substrate) that is associated with proton leak. In liver mitochondria, this proportion can be about 10-15%, and in muscle mitochondria - about half of mitochondria. Contribution of proton leak in tissue of cold-blooded animals' mitochondria is fewer than in warm-blooded ones [34-37]. These facts show that the uncoupled respiration, as a thermogenic factor can relate to thermogenesis.

Intensive oxidation of NADH in mitochondrial suspension of brown fat shows the presence of NADH-permeable mitochondria. Such populations of mitochondria also have succinate and cytochrome oxidases [17], i.e. all components of the mitochondrial respiration chain system for intensive oxidation of substrates along uncoupling way. In tissue conditions, use of different inhibitors of mitochondrial respiratory chain, it was shown [27] that the main part of respiration is detected as a proton leak. It seems that in BAT under conditions close *in vivo*, proton leak is very active.

It is generally known that BAT has a small functional significance in organisms due to low mass. In addition, isolation and study of mitochondria from this tissue is not so simple, since it is very rich in fatty acids and thermogenin, causing a double uncoupling effect on mitochondrial respiration. However, its role is to warm the brain of small mammals with the heat of this thermogenic organ [36, 37]. It is also a good target for elucidating the mechanism of tissue and mitochondrial thermogenesis.

The above-mentioned difficulties of working with BAT mitochondria may lead some researchers to be mistaken that BAT mitochondria can be damaged by methodical techniques. On the other hand, nature itself in the course of evolution could have done certain damage in mitochondrial membranes for the purposes of thermogenesis. It can be assumed that nature itself has learned to do it.

If a researcher is dealing with a population of mitochondria with a high membrane permeability and uncoupled respiration, then this population of mitochondria should still have ATPase activity. Under experimental conditions, isolated mitochondria exhibit such activity under the action of uncouplers on mitochondria due to their increased proton conductivity in the inner membrane. However, ATPase can also be activated in other conditions or damage to mitochondrial function.

In the literature, it was previously noted that in addition to uncoupled respiration, muscular mitochondria [38-40] have uncoupled Mg^{2+}-ATPase activity and is subsequently shown in BAT mitochondria and other tissues [40]. According to obtained results, this enzyme is uncoupled with the system of phosphorylating mitochondria, since it capable to hydrolyze intracellular ATP [39, 40].

The function of ATPase in the cell has been discussed in the literature [39, 40]. Saks [40] is inclined to think that this enzyme exists in the tissue condition, however it cannot cause ATP hydrolysis *in vivo*. This ATPase is blocked by function of creatine phosphokinase that rapidly generates creatine phosphate from ATP. Saks considers [40] that ATP is not a transport form of energy within the muscular tissue. In addition, it was found that the blockers of ATPase activity are special proteins [41, 42]. It should be noted that in brown fat there is a very developed creatine-phosphate system [23] that quickly turns ATP into creatine phosphate. It is main transported form of energy in other tissues and that may be in BAT where there is a uncoupled mitochondrial ATPase.

Results of research obtained by Blancher [11] directly related to the clarification of the existence of uncoupling mitochondria in brown fat. It is necessary to show more specifically the two mitochondrial populations. Experiments of polarographic studies on mitochondrial respiration have shown that in hypertonic medium (0.7 M (700 mOsm) sucrose) dehydration of the phosphorylating mitochondria of brown fat occurs as they stop responding to the addition of ADP or uncoupler of 2,4-DNP. However, under these conditions, a significant rate of uncoupled oxidation of NADH and succinate is maintained. These data are interpreted by the presence of non-phosphorylating mitochondria in the suspension along with phosphorylating ones [17]. The non-phosphorylating group of mitochondria has high membrane permeability and uncoupled respiration is not suppressed in the hypertonic medium.

4. Membrane Studies of Thermogenin and the Role of Fatty Acids

It has been shown that UCP1 activates the permeability to protons in experiments with liposomes. However, it is assumed that this activity in liposomes is due to contamination of UCP1 with fatty acids [19]. However UCP1 itself could cause a high liposome conduction without fatty acid addition. Therefore, in these model systems, factor of contamination must be taken into account. Issues of the ion permeability of mitochondrial membranes and the effect of UCP1 are an important aspect of the proton leak system.

In recent years, a patch-clamp technique has developed that can study the properties of proton leak directly on a piece of brown-fat mitochondrial membrane. Perhaps this method allows solving a number of issues. In particular, this method confirms the need for fatty acids for proton conductivity, possibly as a protonophor [21]. When studying beige fat, it was found that not all cells of this tissue have proton conductivity. This method gives certain data on the heterogeneity of adipocytes and possibly mitochondria [23].

A more cardinal value can be the data, received on the symport of fatty acids and proton via UCP1 [2] as previously mentioned about the antiport of these ions. There data indicate that fatty acids and proton pass through UCP1 and possible pass others substances. Patch-clamp method deals with natural membranes and it may help to obtain new information in this field. These investigations may lead to a certain change in the understanding of the mechanism of proton leak. However, little is known about the value of thermogenin channels in the membrane of these mitochondria. These data would help resolve a number of contentious issues on this problem.

5. Different Mitochondrial Membrane Potential

It is possible to note one more interesting problem that is related to the mitochondrial membrane potential. This parameter can be determined by the ratio of the internal and external concentration of inflowing TPP^+ (tetraphenilphosphonium) cations. The problem is how much the overall membrane potential will change if half of the mitochondria in the suspension is uncoupled because of the high inner membrane permeability. This will reduce the inflowing cation into mitochondria by half, and the total membrane potential according to Nernst's formula will decrease by only 18%. Consequently, even if half the amount of mitochondria destroys, this results in a small decrease in the common membrane potential. Perhaps, therefore, by measuring the membrane potential, researchers may not always notice the complexity of this method of studying of mitochondria.

Under a light microscope, at favorable conditions, it was found that approximately half of cardiac mitochondria do not have a membrane potential [17]. Under these conditions, the value of total membrane potential may decrease by approximately 18%, which can be estimated as an insignificant deviation from the expected values. In the existing literature, there is much information about the mitochondrial heterogeneity, including the membrane potential [45-47].

Moreover, what if the nature itself has learned to damage mitochondria? It is possible to damage a certain amount of mitochondria in a cell, but with benefit for the cell. The biological cell is full of heterogeneous mitochondria and the function of many ones is not yet clear.

The foregoing study shows that BAT mitochondria exhibit a number of functional features that generally do not meet the existing publications of proton leak. More than half the amount of BAT mitochondria may not have a membrane potential and carry out uncoupling respiration for heat production. These loosely coupled mitochondria oxidize succinate and NAD-dependent substrates, as well as uncoupled oxidize NADH. In addition to these functions, they have Mg^{2+}-dependent ATPase. Therefore, in the course of presenting this review, a new viewpoint was gradually defined the progress in the investigations of proton leak in BAT mitochondria, and the certain results in this plan were brought into agreement with this view.

6. Discussion

Proton leakage appeared among the researchers as the main conception of biological thermogenesis, since uncoupling proteins (UCPs) were found in many tissues. Thermogenin of BAT was considered as an uncoupling protein localized in inner membrane of all mitochondria of this tissue [9, 19]. In this review it is reported that the former model of mitochondrial heat production [9] has little opportunity and may take about 20-50% of the total bodily metabolism. This share of metabolism should be significantly higher if to compare the heat and cold-blooded organisms that differ in the metabolism level up to 10 times [16, 17]. It is possible that up to 90% of the metabolism is spent on heat production and only 10% is spent on useful needs. The study of brown fat may allow to reveal the full mechanism of biological thermogenesis, since it is brown fat that is fully adapted for thermogenesis. The previous proton leak model with one population of loosely coupled mitochondria may have certain limitations. In particular, it did not explain a weak expression of proton leakage during NAD-dependent substrates oxidation as well as oxidation of added NADH in BAT mitochondria is not explained by this model.

Only in the process of additional study of various oxidation substrates in the BAT mitochondria, it was revealed the utilization of the different substrates, including NADH, along the uncoupled pathway [15].

In order to interpret the stored data on the features of proton leakage in BAT mitochondria, another concept was proposed that allows the existence of a separate subpopulation of mitochondria. This subpopulation has high membrane permeability for the oxidation of succinate, NADH and NAD-dependent substrates (with the addition of coenzyme NAD). All these oxidation reactions are significantly suppressed in the presence of GDP. Here, oxidation of fatty acids is not considered, so proton leakage goes in mitochondria without these acids. Certainly, there may be impurities of fatty acids, but serum albumin was used in experiments. In principle, fatty acids in the proposed concept are not an important factor [48].

Attention should also be paid to the results obtained in the BLM membrane pores formed by thermogenin. Previously, no reports of high conductivity pores in model membranes have been reported in the literature [29]. The possibility of such large pores in the BAT mitochondrial membrane is quite acceptable, since pyridine and adenine nucleotides pass freely through the mitochondrial membrane. At the moment, not all aspects of mitochondrial membrane permeability remain unclear.

Over time, there were reports of the participation of proton leak in addition to thermogenesis involved in various

processes [47, 48]. Also, many experiments have shown the importance of these proteins in aging, in neutralizing ROS compounds in diseases of mitochondrial origin [42, 43 51 52]. These processes should also be involved in metabolic and energy processes that may occur in the uncoupled system.

7. Conclusions

Regarding to brown fat, it is commonly believed that its main function is thermogenesis by UCP1 function in all mitochondria of this tissue. Under the influence of accumulated data, we make a certain addition to the existing scheme of thermogenesis. In particular, it is considered the possibility of functioning of the uncoupling mitochondrial subpopulation in brown fat as the proton leak and thermogenesis. At the same time in the tissue, ATP synthesis is occurred in coupled subpopulation of mitochondria that energizes the endergonic processes of cellular life.

Abbreviations

BAT - brown adipose tissue, ADP-adenosine diphosphate, ATP-adenosine triphosphate, BLM-bilipid membrane, GDP-guanosine diphosphate, NADH-nicotinamide adenine dinucleotide reduced.

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