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Hyperactivation of Succinate Dehydrogenase in Lymphocytes of Newborn Rats

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Abstract—We measured the activity of mitochondrial succinate dehydrogenase (SDH) within cells, in media with near-physiological composition, in lymphocytes immobilized in a blood smear on glass. SDH activity was studied in newborn rats characterized by natural hyperadrenergic status and also in adult animals injected with epinephrine. In most newborns very high activities were recorded, which exceeded the activities in adults at rest 7-8-fold or 3-fold according to the conventional calculation, or more than 30- and 6-fold according to our more precise calculation. The findings support our concept about a selective interaction between adrenergic stimulation and oxidation of succinic acid. According to this concept, epinephrine and norepinephrine specifically activate oxidation of succinic acid, whereas blood micromolar concentrations of the latter stimulate the release of catecholamines (the receptor-mediated signaling effect). This interaction is half of a substrate–hormonal regulatory system responsible for connection of vegetative nervous system with oxidation in mitochondria of the innervated organs. The increase in succinate oxidation by catecholamines includes activation of the faster pathways of succinate generation than the complete Krebs cycle, in particular, the glyoxylate cycle that is shown in the newborn rats in the present study.

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Key words: newborns, epinephrine, succinate dehydrogenase, succinic acid, glyoxylate cycle

Physiological conditions of newborns are markedly shifted towards the prevalence of sympathetic (adrenergic) regulation compared to adult animals [1]. This regulation is expressed much stronger than in adults under conditions of epinephrine-induced physiological excitement and seems to be a hyperactivation, or the acute phase of stress. Stimulation of physiological functions by epinephrine includes selective activation of succinate dehydrogenase (SDH) [2-5]. Succinic acid (SA), which is a well-known substrate of oxidation, has also been shown to be a signaling molecule stimulating the release of epinephrine and norepinephrine, and this connects the state of mitochondria with activity of the sympathetic half of the vegetative nervous system [6-9]. A signaling connection also exists between the metabolism of mitochondria and the parasympathetic half of the nervous system through interaction of α-ketoglutarate and acetylcholine [10, 11]. These interactions can be considered as a novel, substrate–hormonal, regulatory physiological system [12]. The proposed concept is supported by the discovery of receptors to SA and α-ketoglutaric acid [13]. The detection of a bidirectional metabolic connection of oxidative processes in mitochondria with the nervous regulation is a new contribution to studies on the physiological problem of the nervous system's information about conditions of the regulated organs.

The signaling effect of SA manifests itself at micromolar concentrations corresponding to its blood level. Some data suggest a greater contribution of SA to metabolism in animal and human newborns than in adults. In babies from birth to one month of age, a markedly higher amount of SA is produced than in humans after age 18 years. The urine concentration of succinate/creatinine is 197 mM/M in newborns compared to 7.7 mM/M in adults [14]. The plasma content of SA in newborn rats is 400 μM, and in adult rats it is 50 μM or even only 5 μM [13, 15]. The mitochondrial respiration in rats during the first three days of life is stimulated 15-fold by the usual substrate concentration of SA (5 mM), whereas all other

Abbreviations: ES) endogenous substrates; MA) malonic acid; NBT) Nitro Blue Tetrazolium; SA) succinic acid; SDH) succinate dehydrogenase.

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substrates stimulate respiration only 1.5-2-fold [15]. Other substrates in micromolar concentrations do not activate respiration, whereas even nanomolar concentrations of SA retain a stimulating effect and 500 μM SA stimulates respiration 7-fold. These data seem to suggest an unusually high activity of SA in newborns.

The goal of the present work was to compare the SDH activity in newborn and adult rats in mitochondria within the cell, in media of near-physiological composition, and in lymphocytes immobilized in a blood smear on glass. The retention of structural organization and selection of medium composition allowed us to much better reveal in such preparations the activation of SDH in the body by injected or endogenous epinephrine compared to isolated mitochondria: 200-500 instead of 20-40%, respectively [16, 17]. The detection by this approach of the SDH activation in newborns is promising for extending our group's concept about a selective interaction between adrenergic stimulation and oxidation of SA, which includes activation of faster pathways of SA production than the full Krebs cycle [2, 17].

MATERIALS AND METHODS

Two-to-three-day-old rats from two litters (n = 13) and adult male Wistar rats (n = 12) with body weight of 180-200 g were obtained from the vivarium of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences. Sacrifice was performed with CO_2. Two groups of adult animals were studied: intact rats (n = 6) at rest and those exposed to epinephrine for 30 min (n = 6) in the physiological stimulating dose of 25 μg/100 g body weight injected intraperitoneally. All newborns were studied without additional exposures.

The SDH activity was determined cytochemically by reduction of Nitro Blue Tetrazolium (NBT) [18-20], but the composition of the incubation media was strongly changed because the routine cytochemical protocols do not correspond to modern conditions of manipulations with mitochondria [16]. From blood taken upon decapitation, thin smears were made on glass, which were dried in air, fixed for 30 sec in 60% acetone, and then incubated for 60 min at 37°C in the basic medium containing NBT (1 mg/ml). Samples with addition of 5 mM malonate and 5 mM succinate were also studied. Reagents used were as follows: KCl of especial purity (Reakhim, Russia); succinic and malonic acids (Sigma, USA); KOH (Reanal, Hungary); Nitro Blue Tetrazolium (Dudley, Germany). The SDH activity was measured by the mean formazan-stained area determined by computerized video microscopy on images of 30 lymphocytes. The SDH activity was calculated using both the routine method based on the mean formazan-stained area in the presence of SA ("total activity") and our modification with subtracting the staining in the absence of SA (on endogenous substrates (ES)) in the presence of malonic acid (MA), which is an inhibitor of SDH. This calculation allowed us to take into account the contribution of endogenous SA to oxidation and, thus, is nearer to the real activity of SDH in mitochondria ("true activity"). In cases of a strong decrease in the total activity of SDH, MA did not lower but rather enhanced the staining on ES in the samples. We think this to be caused by an increased production of superoxide at a very weak influx of electrons from SA or NAD-dependent substrates [21-23]. Such a situation is due to MA and does not occur in its absence. Therefore, on calculating the true activity in such cases we subtract from the parameter values in the presence of 5 mM SA values of the sample on ES. The results were processed using parametric tests. Mean values, standard deviations, and errors of the means were calculated for the groups of animals. The significance of differences was evaluated using Student's t-test.

RESULTS

Figure 1 presents three parameters of NBT reduction in lymphocytes on blood smears from the intact adult rats and from the adult rats treated with epinephrine. The first parameter of the NBT reduction on endogenous substrates and the second parameter in the presence of malonate are in the limits of 0.50 μm^2, which indicates the absence of a contribution of SA to oxidation. Values of the third parameter, which shows the SDH activity in the presence of SA, are different in these groups. The SDH activity in the presence of SA in the intact rats was only

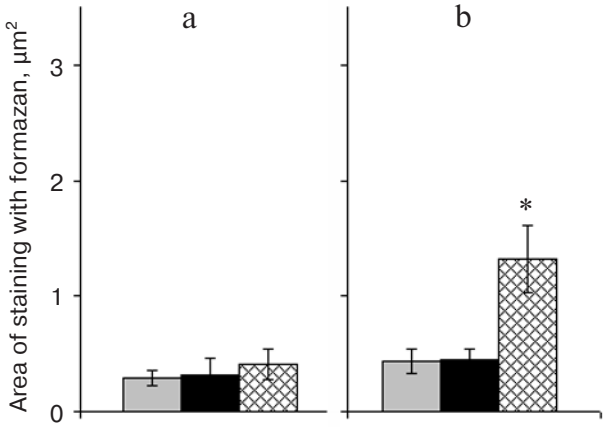

Fig. 1. Nitro Blue Tetrazolium reduction in lymphocytes of intact adult rats at rest (n = 6) (a) and rats treated with epinephrine for 30 min (n = 6) (b). Here and in Figs. 2 and 3, gray columns show NBT reduction on endogenous substrates; black columns, NBT reduction on addition of 5 mM malonate; hatched columns, NBT reduction on addition of 5 mM succinate. Mean values and standard deviations for the group are presented. * Differences relative to the intact animals are significant at $p < 0.001$.

20% higher than the reduction on ES, and the treatment with epinephrine resulted in 2-3-fold increase in the activity of SDH up to the value of 1.4 μm^2.

In most of the newborn rats, all parameters of NBT reduction were markedly higher than in the adult rats even after treatment of the latter with epinephrine. Figure 2 presents the corresponding data for the greater litter. The data are arranged from the highest activity of SDH according to its lowering. In most of the newborns the SDH activity in the presence of SA was strongly increased compared to the adult rats even when the latter were injected with epinephrine. In the first four newborns, the SDH activity was 7-8-fold higher than in the adult rats at rest. The reduction value on ES in these newborns was 3-4-fold higher than in the epinephrine-treated adults. Note that MA decreased this reduction by 30 and even by 90%. This shows that endogenous SA should substantially contribute to oxidation as a result of a pronounced adrenergic activation in the newborns' body even without additional stimuli. Note that, along with the group with the hyperactive SDH, the enzyme in some newborns was lower, close to its activity in the epinephrine-treated adults. These newborns with the lower activity of SDH also displayed a low NBT reduction on ES. The effect of MA in these animals was sharply different: instead of decreasing the staining, its addition strengthened it 2-5-fold. We observed this effect of MA in different cases of SDH inhibition and consider it as the NBT reduction by superoxide, the generation of which increases with a decrease in the SA concentration or inhibition of SDH.

Based on specific features of the NBT reduction, we subdivided the newborn rats from the two litters into three subgroups (Fig. 3): the first subgroup with a hyperactive SDH, the second subgroup with the SDH activity similar to that in the epinephrine-treated adult rats, and the third subgroup with a markedly lower SDH activity. These newborn subgroups were different not only in the SDH activity on oxidation of succinate but also in its ratios to two other parameters: reduction in the presence of malonate and on endogenous substrates. The high activity of SDH in the first subgroup was associated with a high level of reduction on ES and MA decreased it two thirds. In the second subgroup, the reduction on ES was lower than in the first subgroup, MA did not decrease it, and the ratios between all parameters were similar to those in the epinephrine-treated adult rats. The lowest SDH activity was associated with the low reduction on ES, and MA increased it 2-fold. We have also recorded a similar combination of these three parameters in humans. Our earlier data allowed us to characterize the first and second subgroups as hyperactive and active, and these states were caused by endogenous epinephrine and norepinephrine, whereas the third subgroup was inactive.

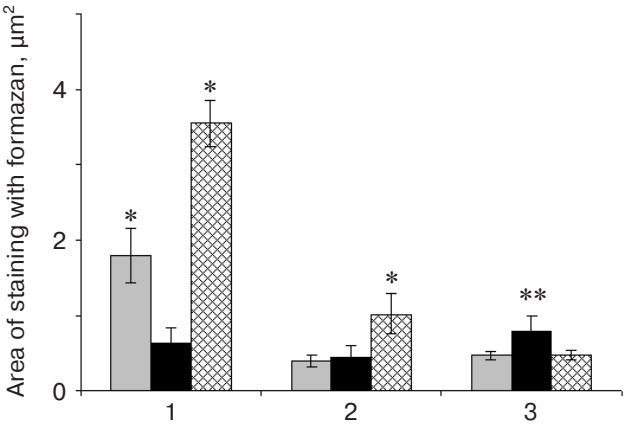

Fig. 3. Changes in the NBT reduction in lymphocytes in the blood smears from newborn rats (n = 13) subdivided into three subgroups according to the SDH activity in the presence of 5 mM SA, reduction on ES, and the influence of 5 mM MA on this reduction. The newborn subgroups: subgroup 1 (n = 4) was characterized by hyperactive SDH, high reduction on ES strongly inhibited by MA; subgroup 2 (n = 4) had SDH activity similar to that in the epinephrine-treated adult rats, and the low level of NBT reduction on ES insuppressible by MA; subgroup 3 (n = 5) was characterized by the low activity of SDH, the low level of NBT reduction on ES increasable by MA. Mean values and standard deviations are presented for each subgroup. *, ** Differences with respect to the adult animals are significant at $p < 0.001$ and $p < 0.05$, respectively.

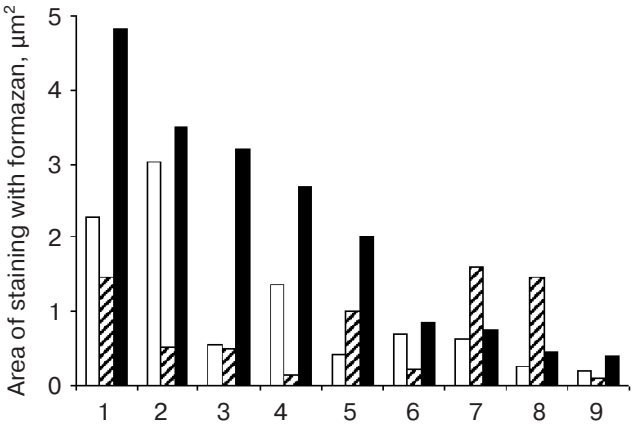

Fig. 2. Individual parameters of NBT reduction in lymphocytes in the blood smears from three-day-old rats of the same litter (n = 9).

Figure 4 presents the above-described findings for the adult and newborn rats obtained as a result of the more precise calculation of the SDH activity with subtraction of the reduction on ES in the presence of MA (designated as "true activity") (Fig. 4a). The differences between the adult and newborn groups in this parameter are more pronounced than in the total activity of SDH. Figure 4b presents the ratio of the true SDH in each group to its value in the adult rats at rest. The injection of epinephrine into adult animals activated SDH nearly 10-

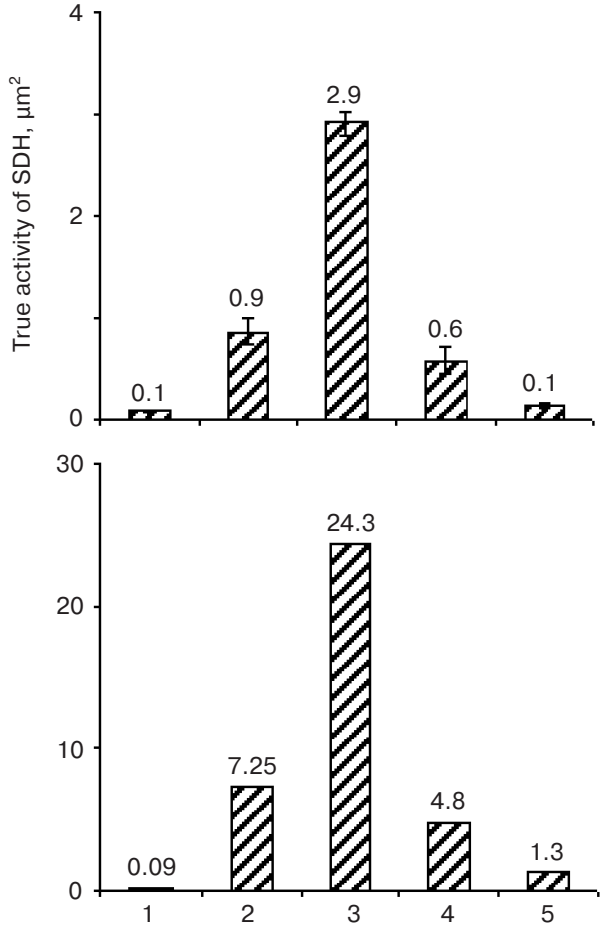

Fig. 4. The "true activity" of SDH in the adult and newborn rats: a) mean true activity in each group; b) ratio of the mean true activity to this parameter in the adult intact rats at rest. *1*) Adult intact rats; *2*) adult epinephrine-treated rats; *3-5*) newborn subgroups characterized by hyperactive, active, and poorly active SDH.

fold. Activation by endogenous catecholamines in the first subgroup of the newborns was more than 30-fold, in the second subgroup it was 6-fold, and in the third subgroup it was nearly 2-fold higher than in the adult animals at rest. Thus, in all newborns the true activity of SDH was higher than in the intact adult rats.

The subdivision into three groups by the totality of definite parameters of newborn rats even from the same mother is consistent with the known heterogeneity of the population and the subsequent selection of more adapted individuals. This is specific for all mammals giving birth to several newborns. In our vivarium, rats give birth to 4-12 young ones. Some of them die two-to-three days after birth, and the mother rat herself tramples them down. The more healthy and active individuals survive. It seems that the subgroup with the high SDH activity corresponds to more physiologically sound or more mature individuals and the subgroup with the low SDH activity includes the weak individuals with delayed development.

DISCUSSION

Our findings show new material on an increase in SDH activity in the body under conditions of enhanced adrenergic influence, which has been shown earlier during the acute phase of stress caused by release of endogenous epinephrine [24] or by injection of its physiologically activating doses [2, 25, 26]. The idea about the prevalence of adrenergic reactions in newborns based on physiological data [1] is also supported by determinations of blood catecholamines [27]. The activation caused by injection of epinephrine or by endogenous catecholamines shown in the present work is close in amplitude to the strong catecholamine-induced activation of physiological processes in the body and is significantly greater than effects observed on isolated mitochondria or on the routine cytochemical determination. This is explained by retention in our experiments of the initial state of rest, which is lost on the isolation of mitochondria or placing lymphocytes into a non-physiological medium. This is confirmed by our recording of a virtually inactive SDH in the rats at a full-value rest. This state is latent or inactive but in no case an inhibited one, which we showed separately using SDH activators. This state was clearly seen in lymphocytes from the adult rats at rest: notwithstanding the addition of SA, there was virtually no response of SDH, as if it "slept" or was switched off (Figs. 1 and 3). This is corresponds well to adrenergic influences in the body: they are switched off at rest and switched on upon activation. Just the retention of the rest state under conditions of our experiment allowed us to more clearly reveal the interrelation between the SA oxidation and epinephrine effect, and we have designated this interrelation as a sympathetic half of the substrate–hormonal system responsible for ATP synthesis during activity.

The switching from the state of rest to the epinephrine/norepinephrine stimulated active type of metabolism has been shown to be associated with an increased contribution to oxidation of the accelerated generation of SA through transamination of glutamic and oxalacetic acids [2]. These reactions form the so-called truncated Krebs cycle [28]. We think that it increases the contribution of the most powerful oxidation of SA to the energy provision under conditions of great expenditure of ATP.

The study performed on newborns has discovered another metabolic pathway for achievement of the same result. The experiments performed on the same newborn rats have revealed in them key glyoxylate cycle enzymes, isocitrate lyase intensively producing SA and malate synthase [18]. Up to now the classic concept has been that this cycle exists only in microorganisms and plants but is absent in higher animals. Still on discussing with Krebs, M. N. Kondrashova supposed that the glyoxylate cycle of SA generation is switched on in animals on excitement caused by release of epinephrine [18]. Therefore, failures in its detection in animals were caused by studies per-

formed on animals at rest, which was sometimes aggravated by a fat-enriched diet. There were some subsequent reports about detection of the glyoxylate cycle under conditions other than rest, in particular, during starvation [29], stress [30], and some other states [31]. As mentioned in the introduction, the state of newborns is another example of adrenergic regulation in the body, which includes the SDH activation and increased influx of SA. In the above-listed cases, such as acute stress, long-term starvation, and milk diet of newborns, the production of SA was provided for by decomposition of lipids, as occurs in seeds of oil plants. The hyperactivation of SDH in the newborns revealed in our work can be also caused by the increase in SA concentration due to activation of the transaminase and glyoxylate oxidation cycles and also by the epinephrine-induced activation of SDH.

A considerably higher level of SA in newborns than in adults can be used for both maintaining intense energy provision in mitochondria and signaling activation of release of catecholamines, which are dominating hormones during early ontogenesis. Thus, SDH is an enzyme on which substrate–hormonal partners represented by succinic acid and catecholamines interact in mitochondria.

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