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Effect of calcium overload on key dehydrogenases in heart mitochondria

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The effect of supra-physiological Ca^{2+} concentration on the activity of Ca^{2+}-sensitive tricarboxylic cycle dehydrogenases was investigated with the aim to determine if a very fast decrease in mitochondrial NAD(P)H content induced by Ca^{2+} overload may be caused by changes in activity of these dehydrogenases. We showed that pyruvate and 2-oxoglutarate dehydrogenases in heart mitochondria were stimulated by Ca^{2+} in the physiological range of concentration but were not affected by Ca^{2+} overload. In complete contrast, Ca^{2+} affected isocitrate dehydrogenase (ICDH) only in the supra-physiological range of concentration. The effect of Ca^{2+} on ICDH depended on isoform and substrate concentration. We showed that in heart mitochondria NADP-ICDH could be from 100 to 350-fold more active than NAD-ICDH. The ratio of their activities decreased with an increase in Ca^{2+} concentration, because NADP-ICDH was inhibited by Ca^{2+}, while NAD-ICDH was activated. We concluded that among the key mitochondrial dehydrogenases only ICDH isoforms might potentially contribute to Ca^{2+}-overload-induced changes in NAD(P)H concentration.

Key words: heart mitochondria, calcium ions, NAD(P)-dependent dehydrogenases, NAD(P)H

INTRODUCTION

Mitochondria belong to a long list of intracellular targets affected by calcium overload which occurs under various pathological conditions [1–3]. We have previously shown that an increase in extramitochondrial Ca^{2+} concentration above the physiological level (i.e. 1 μM) has multiple negative effects on mitochondrial function [4]. The respiratory chain was more inhibited by Ca^{2+} overload in the case of oxidation of NAD-dependent substrates in comparison with succinate [4]. Ca^{2+} overload induced a substantial decrease in the amount of NADH, a key substrate of the respiratory chain, although it did not inhibit the activity of Complex I directly. In addition, the decrease in NADPH content was also caused by Ca^{2+} overload [5, 6].

The aim of this study was to examine whether the calcium overload-induced decrease of mitochondrial NAD(P)H is determined by Ca^{2+} effects on the NAD(P)-dependent tricarboxylic acid cycle (TCA) dehydrogenases. In the mammalian cells, three dehydrogenases that provide a substantial part of NADH to the respiratory chain are activated at micromolar and submicromolar Ca^{2+} concentrations: pyruvate dehydrogenase (PDH) complex [7], NAD^+-dependent isocitrate dehydrogenase (NAD-ICDH) [8] and 2-oxoglutarate dehydrogenase (OGDH) [9]. Ca^{2+} activates PDH complex indirectly [8] by lowering K_m of phospho-PDH-phosphatase subunit for magnesium ions [7, 8, 10]. The other two dehydrogenases, NAD-ICDH and OGDH, are activated by calcium ions directly [9, 11] by lowering K_m values for their substrates. NAD-ICDH is activated by Ca^{2+} at concentrations from 5 to 20 μM [11, 12], and OGDH is activated by Ca^{2+} with a K_m of about 1 μM [9, 13]. The crucial importance of the response of these essential dehydrogenases to Ca^{2+} within the physiological range of concentration for the regulation of TCA was established long ago (reviewed in [14]). However, there is still the lack of data about their response to Ca^{2+} in the range of higher concentrations relevant to many pathological states of the cell. Mitochondria also contain $NADP^+$-dependent isocitrate dehydrogenase (NADP-ICDH) [15–17], but little is known about the sensitivity of this isoform to calcium ions. The aim of this study was to compare the activity of PDH, OGDH and both isoforms of ICDH in the range of physiological Ca^{2+} concentrations used in previous studies [8–13] to that at much higher supra-physiological Ca^{2+} levels. These dehydrogenases are essential regulatory sites of oxidative phosphorylation, therefore the obtained information contributes to the understanding of Ca^{2+}-induced impairment of mitochondrial energy metabolism.

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MATERIALS AND METHODS

Mitochondria were isolated from the heart of male Wistar rats by differential centrifugation, and the protein concentration was estimated by a modified biuret method as described earlier [4–7]. The animals were killed according to the rules defined by the European Convention for the protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (License No. 0006). Mitochondria were suspended in a buffer containing 180 mM KCl, 20 mM Tris-HCl, 3 mM EGTA (pH 7.3) and stored on ice.

The activities of PDH, OGDH, NAD-ICDH and NADP-ICDH in mitochondria (dissolved by adding 0.2% (v/v) Triton X-100) were measured spectrophotometrically by following the rate of NAD^+ (or $NADP^+$) reduction at 340 nm in a medium containing 30 mM Tris-HCl, 5 mM KH_2PO_4, 135 mM KCl, 10 mM NaCl, 1 mM EGTA, 5 mM NTA, 2 mM NAD^+ (or 1 mM $NADP^+$ for NADP-ICDH assay), 2 μM rotenone, mitochondria (0.1–0.25 mg protein) and/or 0.875 mM $CaCl_2$ (1 μM free Ca^{2+}) and 5.17 mM $MgCl_2$ (1 mM free Mg^{2+}) or 1.3 mM $CaCl_2$ (10 μM free Ca^{2+}) and 4.95 mM $MgCl_2$ (1 mM free Mg^{2+}) or 1.5 mM $CaCl_2$ (30 μM free Ca^{2+}) and 4.8 mM $MgCl_2$ (1 mM free Mg^{2+}), pH 7.2 37°C [3–6]. For the assay of PDH and OGDH activity the medium was supplemented with 1 mM DTT, 1 mM thiamine pyrophosphate, 1 mM ADP. The concentrations of pyruvate, 2-oxoglutarate and isocitrate are indicated in figure legends. The reaction for assaying PDH and OGDH activities was initiated by adding 0.25 mM CoA and for the NAD-ICDH and NADP-ICDH by addition of substrate. The rate of reactions was linear for 1–1.5 min.

RESULTS

TCA dehydrogenases supply NADH for the respiratory chain in heart mitochondria oxidizing physiological substrates. The stimulation of PDH, OGDH and NAD-ICDH by Ca^{2+} ions within the physiological range of concentration effectively activates mitochondrial respiration and ATP synthesis [14]. However, the mechanism whereby Ca^{2+} overload causes impairment of mitochondrial processes remains not

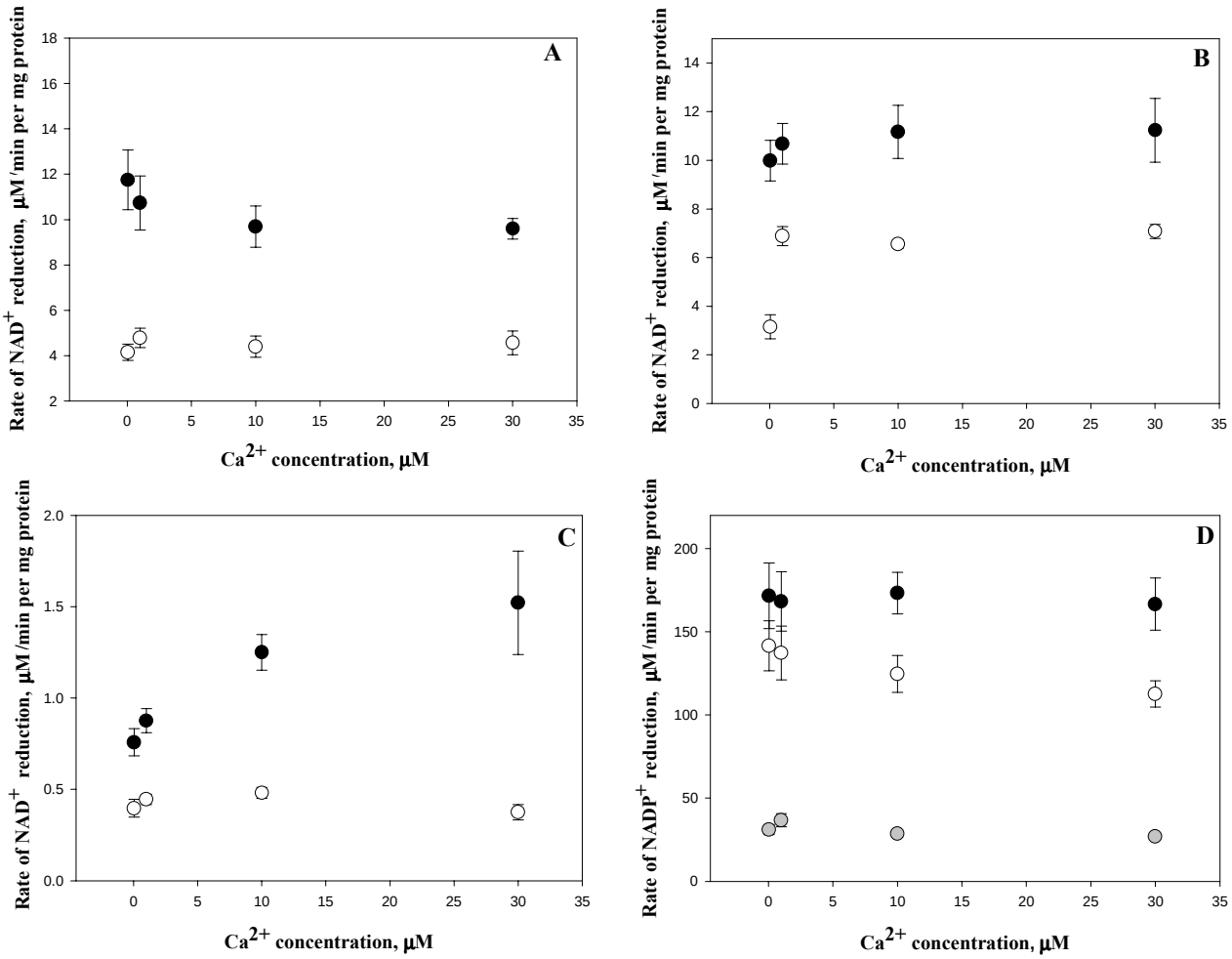

Fig. 1. Dependence of activity of four mitochondrial dehydrogenases on Ca^{2+} concentration in the medium. A – pyruvate dehydrogenase activity (○ – pyruvate + malate 0.1 mM + 0.1 mM, ● – pyruvate + malate 1 mM + 1 mM; n = 5); B – 2-oxoglutarate dehydrogenase activity (○ – 0.1 mM 2-oxoglutarate, ● – 1 mM 2-oxoglutarate; n = 6); C – NAD^+-dependent isocitrate dehydrogenase activity (○ – 0.1 mM D,L-isocitrate, ● – 1 mM D,L-isocitrate, n = 4); D – $NADP^+$-dependent isocitrate dehydrogenase activity (◎ – 0.01 mM D,L-isocitrate, ○ – 0.1 mM D,L-isocitrate, ● – 1 mM D,L-isocitrate; n = 4).

completely understood. We investigated how a supra-physiological Ca^{2+} concentration affects the activity of Ca^{2+}-sensitive tricarboxylic cycle dehydrogenases and NADP-ICDH. To this end, their enzymatic activity was estimated at four Ca^{2+} concentrations: very low (5 nM), "stimulating" (1 μM, the upper physiological limit), Ca^{2+} concentration relevant to cell under different pathologies (10 μM) [12], and a very high Ca^{2+} concentration (30 μM). Bearing in mind that Ca^{2+} concentration in the matrix of energized mitochondria is 2–4-fold lower than outside [18], it is hardly possible that dehydrogenases could be exposed to concentrations of free Ca^{2+} higher than 30 μM.

The obtained results (Fig. 1 A, B, C) about stimulating Ca^{2+} effects on PDH, OGDH and NAD-ICDH are in complete agreement with data reported by other authors [6–8, 19]. PDH activity at a sub-saturating concentration of substrate (Fig. 1A) and 5 nM Ca^{2+} was 4.15 ± 0.36 μM/min per mg protein, and it was by 15% higher at 1 μM Ca^{2+} ($p < 0.05$). The activity of PDH did not change when Ca^{2+} concentration was increased to 10 or 30 μM (4.40 ± 0.46 and 4.56 ± 0.52 μM/min per mg protein, respectively). A tenfold increase in pyruvate + malate concentration in the medium with 5 nM Ca^{2+} (Fig. 1A) led to an almost a threefold increase in the rate of NAD^+ reduction (from 4.15 ± 0.36 to 11.75 ± 1.32 μM/min per mg protein). However, at the saturating concentration of substrate, a higher Ca^{2+} concentration (1, 10 and 30 μM) did not alter PDH activity (Fig. 1A). The tendency of decrease in PDH activity was not statistically significant.

In agreement with others [19, 20] we found that OGDH is the most sensitive target of direct activation by Ca^{2+}. OGDH activity (Fig. 1B) in the presence of sub-saturating substrate concentration (0.1 mM) was 3.15 ± 0.50 μM/min per mg protein in the medium with 5 nM Ca^{2+}. OGDH activity was 2.2-fold higher at 1 μM Ca^{2+}. However, the further increase of Ca^{2+} concentration to 10 and 30 μM had no effect on OGDH activity. OGDH was substantially (from 3.15 ± 0.50 to 9.99 ± 0.83 μM/min per mg protein) activated by the increase of substrate concentration in the medium to 1 mM (Fig. 1B). However, at the saturating concentration of substrate, the sensitivity of OGDH to the change in Ca^{2+} concentration in the medium (from 5 nM to 1 μM as well as from 1 μM to 10 or 30 μM) was only negligible. These results are in line with the established fact that Ca^{2+} ions activate OGDH by lowering K_m for 2-oxoglutarate [13]. Thus, our data (Fig. 1, A and B) show that neither PDH nor OGDH is inhibited at a supra-physiological concentration of Ca^{2+} ions.

The dependence of NAD-ICDH activity on Ca^{2+} concentration in the medium containing different concentrations of D,L-isocitrate is shown in Fig. 1C. In line with the reported NAD-ICDH $K_{0.5}$ values for Ca^{2+} (10–25 μM) [8, 11], a significant activation of NAD-ICDH in our experiments was observed only when Ca^{2+} concentration in the medium increased from lower values to 10 μM (at 0.1 mM substrate). The rate of NAD^+ reduction increased by 8% (0.40 ± 0.03 and 0.48 ± 0.03 μM/min per mg protein at 1 μM Ca^2 and at 10 μM Ca^{2+}, respectively; $p < 0.05$). However, exposure of mitochondria to 30 μM Ca^{2+} caused an inhibition of the enzyme; the rate of reaction was reduced by 22% (0.38 ± 0.04 μM/min per mg protein at 30 μM Ca^{2+}, $p < 0.05$).

The activity of NAD-ICDH at 5 nM Ca^{2+} increased twofold with an increase in D,L-isocitrate concentration from 0.1 mM to 1 mM (Fig 1C). The increase of Ca^{2+} concentration from 5 nM to 1 μM led to a 16% increase in the rate of NAD^+ reduction (from 0.76 ± 0.07 to 0.88 ± 0.06 μM/min per mg of protein, $p < 0.05$). In the medium with 10 μM Ca^{2+}, the rate of reaction was by 43% ($p < 0.05$) higher than at 1 μM Ca^{2+}. The activity of enzyme was not affected by the increase of Ca^{2+} concentration from 10 to 30 μM. These data confirm the results of other authors that calcium ions activate NAD-ICDH at a higher concentration than the one needed for the activation of PDH and OGDH [8, 21].

NADP-ICDH is present in mitochondria, cytosol and peroxisomes, however, the biological role and characteristics of this isoform are neither clearly understood nor well investigated [17]. The reported K_m values of NADP-ICDH for D,L-isocitrate vary from 0.005 to 0.02 mM [8, 11], therefore the dependence of NADP--ICDH activity on Ca^{2+} concentration was determined at three different concentrations of substrate – 0.01, 0.1 and 1 mM (Fig. 1D). Increasing the substrate concentration at 5 nM Ca^{2+} from 0.01 mM to 0.1 mM led to a 4.5-fold increase of the rate of reaction, but the rate was not statistically different at 0.1 and 1 mM of substrate. An increase of Ca^{2+} concentration from 5 nM to 1 μM did not affect the rate of reaction in the whole range of substrate concentrations. NADP-ICDH activity was inhibited by an increase in the Ca^{2+} concentration from 5 nM to 10 or 30 μM, but this effect was more pronounced at a lower substrate concentration. The reaction rate at 10 and 30 μM as compared to that at 1 μM Ca^{2+} was respectively diminished by 22% ($p < 0.05$) and 26% ($p < 0.05$) at 0.01 mM of substrate. At higher (0.1 mM) substrate concentration decrease in the rate was smaller: at 10 μM Ca^{2+} the rate was significantly by 12% and at 30 μM Ca^{2+} – by 20% lower than at 5 nM Ca^{2+}. The NADP-ICDH activity was not altered by Ca^{2+} when substrate concentration was increased to 1 mM.

DISCUSSION

Nicotinamide adenine dinucleotides, $NAD(P)^+$ and NAD(P)H, are among the most essential cell metabolites. The $NAD(P)H / NAD(P)^+$ ratio reflects the balance between energy-supplying and energy-consuming processes in the cell under physiological conditions. It has been recently shown that oxidation and depletion of NAD(P)H is a primary event in the stress signal pathways [22]; however, the mechanisms whereby various stress factors cause NAD(P)H depletion are not established. Ca^{2+} ions are multifunctional second

messengers which, besides numerous physiological roles, are also involved in the stress response related to the disturbance of normal Ca^{2+} homeostasis, resulting in Ca^{2+} overload [1–3]. The essential role in cellular response to Ca^{2+} overload is played by mitochondria behaving as temporary cellular safety devices in situations of Ca^{2+} emergency [3]. However, rapid accumulation of excessive Ca^{2+} amounts by mitochondria is followed by dysfunction of their respiration and ATP synthesis, loss of the membrane potential, opening of the permeability transition pore which is an important determinant of cell death or the pathogenesis of a number of diseases [29]. Ca^{2+} overload-induced and rotenone-insensitive NAD(P)H depletion [5, 6] causes inhibition of respiration [4, 5] and facilitates the opening of the permeability transition pore [30]. Aiming to find out the possible causes of NAD(P)H depletion, we investigated the effect of Ca^{2+} ions on several main suppliers of NADH and NADPH–NAD(P)-dependent dehydrogenases operating in the mitochondrial matrix.

Our results essentially reproduce earlier reported data on the activation of PDH, OGDH and NAD-ICDH by Ca^{2+} ions in the physiological range of concentrations [7–13, 20, 21]. We show that independently of substrate concentration, PDH and OGDH activity is not sensitive to a high concentration of Ca^{2+} ions (Fig. 1, A and B). Thus, the contribution of these two dehydrogenases to Ca^{2+} overload-induced changes in mitochondrial NAD(P)H amount is excluded.

Ca^{2+} overload has an effect only on ICDH, and the obtained data reveal a great difference in the sensitivity of NAD-ICDH and NADP-ICDH isoforms to Ca^{2+} ions (Fig. 1, C and D). A comparison of their activities indicates that in heart mitochondria NADP-ICDH can be from 100 to 350-fold more active than NAD-ICDH, depending on conditions. In complete contrast to PDH and OGDH, Ca^{2+} affected ICDH only in the supra-physiological range of concentration. The effect of Ca^{2+} on ICDH depended on isoform and substrate concentration.

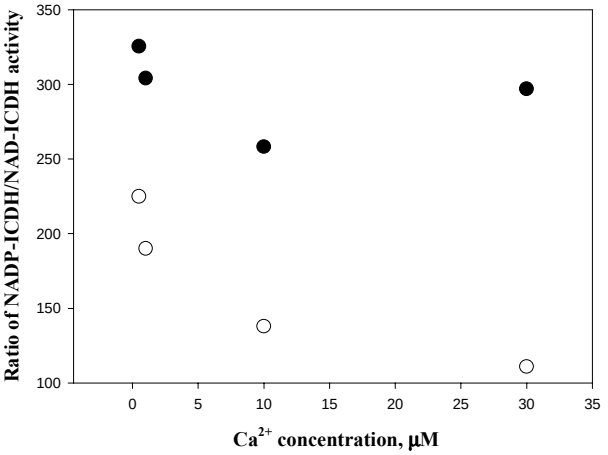

Fig. 2. Dependence of the ratio of NADP-ICDH / NAD-ICDH activity in heart mitochondria on Ca^{2+} concentration in the medium. ● – 0.1 mM D,L-isocitrate; ○ – 1 mM D,L-isocitrate; n = 4.

In the presence of a sub-saturating concentration of substrate, NAD-ICDH was 8% activated by increase in Ca^{2+} from 1 μM to 10 μM, while an increase in Ca^{2+} concentration from 5 nM to 10 μM decreased the activity of NADP-ICDH by 12% (the inhibition was twice stronger at 0.01 mM substrate). In the medium with 1 mM isocitrate, an increase in Ca^{2+} from 1 to 10 μM caused a 43% increase in NAD-ICDH activity whereas NADP-ICDH activity was not altered. The increase of Ca^{2+} from 10 to 30 μM inhibited both NADP-ICDH (by 10%) and NADH-ICDH (by 22%) at a low substrate concentration, but did not change the activity of the isoforms at 1 mM substrate. Activation of NAD-ICDH by 10 μM Ca^{2+} at 1 mM of isocitrate excludes the possibility that the effective Ca^{2+} concentration is decreased by binding to substrate (Fig. 1C).

Thus, our data indicate that Ca^{2+} overload *in vivo* (more relevant to the experimental conditions when the medium contains 1–10 μM Ca^{2+}) should lead to a stimulation of NADH-ICDH and moderate inhibition of NADP-ICDH. Many authors state that, in contrast to PDH and OGDH, NAD-ICDH is activated by a higher than physiological concentration of Ca^{2+} [8, 11]. The biological importance of this fact is not clear, but it is known that under pathological conditions NAD-ICDH activation by Ca^{2+} could improve the supply of substrates to NADH/NADPH transhydrogenase and the respiratory chain. On the other hand, it has been established that NADPH is an allosteric inhibitor of NAD-ICDH [16]. The inhibition of NADP-ICDH could be useful to direct more NADH to the respiratory chain under calcium overload.

The obtained results show that under all experimental conditions NADP-IDH activity markedly, 100- to 350-fold, exceeded NAD-IDH activity (Fig. 2). The differences in the activities of these two isoforms in heart mitochondria were much higher than those reported by some researchers (e.g., a 4–14-fold difference [16]), but very similar to those obtained by others [23]; most probably this could be explained by differences in experimental conditions. The activity and expression of mitochondrial NADP-ICDH isoform is highest in the heart [24], and it is exclusively (95%) located in mitochondria. The necessity for the excess of this enzymatic activity in comparison to NAD-ICDH is not yet clearly defined. The biological role of mitochondrial NAD-ICDH is confined to an irreversible allosterically regulated reaction that supplies reducing equivalents from TCA to the respiratory chain [25]. A structurally and catalytically different NADP-ICDH isoform catalyses the reaction providing NADPH for numerous mitochondrial pathways for biosynthesis and antioxidant defense. Higher levels of mitochondrial NADP-ICDH render the tissues resistance to oxidative damage [26]. In contrast to NAD-ICDH, the reaction catalysed by NADP-ICDH is reversible. There are evidences that, both in the liver and in the heart, NADP-ICDH can operate in the reverse direction [23, 27], thereby generating a substrate cycle important for TCA regulation [16].

In energized mitochondria, nicotinamide nucleotide transhydrogenase maintains a high NADPH / $NADP^+$ ratio,

therefore the concentration of NADP-ICDH substrate $NADP^+$ under physiological conditions is much lower than the concentration of NAD-IDH substrate NAD^+ (the NADPH / $NADP^+$ ratio in heart mitochondria is >50 [28]). Consequently, the great differences in the activities of mitochondrial ICDH isoforms should be compensated by the concentrations of their substrates. Our data show that with an increase in Ca^{2+} concentration the ratio of NADP-IDH and NAD-IDH activity under optimal conditions decreases (Fig. 2). This tendency is clearer at 1 mM substrate, because the first enzyme is inhibited by Ca^{2+}, while the latter is activated (Fig. 1, C and D). However, inhibition of respiration by Ca^{2+} overload is followed by a drop in the membrane potential [4]. Under these circumstances the nicotinamide nucleotide transhydrogenase is not able to maintain a high NADPH / $NADP^+$ ratio, and the flux through NADP-ICDH might be activated.

The data obtained in this study indicate that among the key mitochondrial dehydrogenases only ICDH isoforms (but not PDH or OGDH) might potentially contribute to Ca^{2+} overload-induced changes in NAD(P)H concentration. However, additional work is required to resolve how Ca^{2+} overload may interfere with the complex network of mitochondrial enzymes involved in energy metabolism and how that disturbs the balance of cellular reduction / oxidation state and affects the pyridine nucleotide pool in response to a variety of challenges.

ABBREVIATIONS

ICDH – isocitrate dehydrogenase; OGDH – 2-oxoglutarate dehydrogenase; PDH – pyruvate dehydrogenase; TCA – tricarboxylic acid cycle

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KALCIO PERKROVOS POVEIKIS ESMINĖMS ŠIRDIES MITOCHONDRIJŲ DEHIDROGENAZĖMS

S a n t r a u k a

Siekdami įvertinti, ar Ca^{2+} perkrovos sukeltas greitas mitochondrijų NAD(P)H kiekio mažėjimas yra susijęs su Ca^{2+} poveikiu Ca^{2+} jautrių trikarboksirūgščių ciklo dehidrogenazių aktyvumui, nustatėme Ca^{2+} jonų poveikį šiems fermentams, kai viršijama fiziologinė Ca^{2+} koncentracija. Rezultatai rodo, kad Ca^{2+} jonai aktyvina širdies mitochondrijų piruvato ir 2-oksoglutarato dehidrogenazes esant fiziologinei Ca^{2+} koncentracijai, tačiau nekeičia jų aktyvumo Ca^{2+} perkrovos metu. Priešingai, Ca^{2+} jonai turėjo poveikį izocitrato dehidrogenazės (ICDH) aktyvumui tik jų koncentracijai viršijus fiziologinę ribą, t. y. Ca^{2+} perkrovos metu. Ca^{2+} jonų poveikis ICDH aktyvumui priklausė nuo fermento izoformos ir substrato koncentracijos. Širdies mitochondrijų NADP-ICDH aktyvumas buvo 100–350 kartų didesnis už NAD--ICDH izoformos aktyvumą. Didinant Ca^{2+} koncentraciją, NADP--ICDH ir NAD-ICDH aktyvumo santykis mažėja, nes Ca^{2+} jonai slopina NADP-ICDH ir skatina NAD-ICDH. Gauti rezultatai leidžia teigti, kad tarp svarbiausių Ca^{2+} jautrių mitochondrijų dehidrogenazių tik ICDH gali turėti įtakos Ca^{2+} perkrovos sukeltiems NAD(P)H koncentracijos pokyčiams mitochondrijose.