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Original article

Pyridine nucleotides regulate the superoxide anion flash upon permeabilization of mitochondrial membranes: An MCLA-based study

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The permeabilization of mitochondrial membranes via permeability transition pore opening or by the pore-forming peptide alamethicin causes a flash of superoxide anion (SA) and hydrogen peroxide production and the inhibition of matrix aconitase. It was shown using the SA probe 3,7-dihydro-2-methyl-6-(4-methoxyphenyl) imidazol[1,2-a]pyrazine-3-one (MCLA) that the substrates of NAD-dependent dehydrogenases, inhibitors of the respiratory chain, and NAD(P)H at millimolar concentrations suppressed or delayed SA flashes. In the presence of added NADH and NADPH, SA flashes were observed only after considerable oxidation of pyridine nucleotides. The production of SA was maximal at NADPH and NADH redox potentials from −315 to −295 mV and from −325 to −270 mV, respectively, depending on NAD(P)H concentration. SA generation supported by NADPH was severalfold greater than that supported by NADH. In intact mitochondria, NADPH- and NADH-dependent SA generation was negligible. Respiratory substrates at physiological or lower concentrations were incapable of suppressing the NADPH-supported SA flash. These data indicate that, in conditions close to pathophysiological, matrix NADPH oxidoreductase(s), presumably, an adrenodoxin reductase in complex with adrenodoxin, can essentially contribute to SA flashes associated with transient or irreversible permeability transition pore opening or membrane permeabilization by another mechanism.

1. Introduction

Mitochondrial permeability transition (mPT) is a Ca^{2+}-dependent permeabilization of the inner mitochondrial membrane (IMM) for ions and solutes of molecular masses less than 1.5 kDa via the creation of an unspecific pore (mPTP) [1]. The molecular composition of mPTP is a matter of debates [2]. However, the regulation of mPTP opening has been well studied. Reactive oxygen species (ROS), oxidants, and thiol reagents facilitate, while cyclosporin A (an inhibitor of peptidyl-prolyl cis-trans isomerase), reduced pyridine nucleotides in the matrix, Mg^{2+}, ATP, and ADP suppress mPTP opening triggered by Ca^{2+} [1]. The inhibitors of adenine nucleotide translocase, which stabilize the carrier either in the cytosolic or the matrix conformation, facilitate or inhibit mPTP opening, respectively [3].

It is well known that the opening of mPTP by Ca^{2+} stimulates the generation of ROS in isolated mitochondria [4–6]. A burst of ROS production occurs in cells in pathologic states that can trigger the mPTP opening [7–9]. The elevation of cytosolic Ca^{2+} and the induction of mPTP are considered to be the main reasons for the activation of ROS production and cell death upon ischemia/reperfusion [10–12]. The transient mPTP opening associated with the excitotoxicity of glutamate in motor neuron-like cells [13], the beta-amyloid-induced suppression of proliferation of neural progenitor cells [14], as well as with Ca^{2+} and oxidative stress [15] also causes a short-term acceleration of SA production (so-called flash) by mitochondria [16].

Several mechanisms were proposed to explain ROS flashes/bursts during mPTP opening and under mPTP-facilitating conditions. It was suggested that Ca^{2+}-activated dehydrogenases, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, can generate ROS with higher rates than unactivated enzymes [17]. It was also assumed that mPTP induces conformational changes in complexes I, II, and III, which accelerates ROS production [4,6,18]. In this case, malic enzyme may generate fuel for ROS production by damaged complex III [4]. An exhaustion of antioxidant systems due to the release of glutathione

Abbreviations: Alam, alamethicin; ADx, adrenodoxin; AR, adrenodoxin reductase; DLD, dihydrolipoamide dehydrogenase; IMM, inner mitochondrial membrane; MDCL, MCLA-derived chemiluminescence; MCLA, 3,7-dihydro-2-methyl-6-(4-methoxyphenyl)imidazol[1,2-a]pyrazine-3-one; mPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; NOX4, NADPH oxidase 4; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; SA, superoxide anion; SOD, superoxide dismutase

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through the mPTP may also contribute to the acceleration of ROS production [5,19]. In the heart, the burst of ROS production may be related to the accumulation of serotonin and its oxidation by monoamine oxidase during ischemia and reperfusion, respectively [20]. However, up to now, the mechanisms underlying the mPTP-related ROS burst remain unclear.

Another mechanism by which mitochondrial membranes can be permeabilized under pathophysiological conditions is the impact of environmental microbial toxins, especially lipophilic and positively charged, on eukaryotic organisms. It is well documented that chronic exposure of humans in Northern Europe to environmental toxins, including pore-forming alamethicin-like peptaibols and fusaricidins, is harmful to the health. Moreover, crude extracts and pure toxins obtained from the dust and colony particles collected in living and industrial buildings can induce dramatic changes in cellular and mitochondrial functions [21–25].

Here we explored possible mechanisms of SA flash in mitochondria upon the membrane permeabilization via mPTP opening and by alamethicin (Alam) using the chemiluminescent probe MCLA. MCLA seems to be a superior SA probe for many applications [26–30]. It is extremely sensitive to SA (hundreds of times more sensitive than lucigenin) [31], though it can also sense $^1O_2$ [32]. Many SA probes, namely, tetrazolium salts, lucigenin, cytochrome *c*, and epinephrine are either unspecific or capable of inducing the additional ROS production in biological systems [28,33–35]. MCLA, presumably, lacks these drawbacks. By contrast to lucigenin [34,36] and oxidized products of hydroethidine (Mito-SOX), ethidium and 2-hydroxyethidium [37], both MCLA and its superoxide adduct are uncharged [32], which makes chemiluminescence independent of the membrane potential. This property seems advantageous in the context of the present study, since the permeabilization of the IMM causes immediate dissipation of the membrane potential. The known drawbacks of MCLA are a rather high spontaneous luminescence in solution [32] and chemiluminescence quenching by sulfur-containing compounds [38]. Both problems can be solved by the selection of appropriate experimental conditions.

Using MCLA in a model of isolated mitochondria, we found that the permeabilization of the IMM via mPTP opening and by Alam causes an SA flash, which is regulated by the redox state of internal and external NADH and NADPH. External (cytosolic) NADPH and internal NADPH oxidoreductase(s) can make a major contribution to the SA flash upon the IMM permeabilization. The pathophysiological relevance of our findings was discussed.

2. Materials and methods

2.1. Materials

Alamethicin, Ampliflu™ Red (Amplex Red), bovine serum albumin (BSA), cytochrome *c* from the equine heart, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), glutamate, α-ketoglutarate, malate, mannitol, 7- dihydro-2-methyl-6-(4-methoxyphenyl)imidazol [1,2-a]pyrazine- 3-one (MCLA), myxothiazol, NADH, NADPH, NAD, NADP, β-oxybutyrate, pig heart isocitrate dehydrogenase, potassium peroxide, pyruvate, rotenone, sucrose, succinate, superoxide dismutase (SOD), and Trizma Base were obtained from the Sigma-Aldrich Corporation (St. Louis, MO; USA). Other chemicals were of analytical grade and were purchased from local suppliers.

2.2. Isolation and purification of rat liver mitochondria

All manipulations with animals before the isolation of the liver were performed in accordance with the Helsinki Declaration of 1975 (revised in 1983) and national requirements for the care and use of laboratory animals. Adult male Wistar rats were killed by cutting the neck after anesthesia with CO_2. Rat liver mitochondria were isolated according to a standard differential centrifugation procedure [39]. The homogenization medium contained 220 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH adjusted to 7.4 with Trizma Base), 1 mM EGTA, and 0.05% BSA. The pellet was washed three times with a medium devoid of EGTA and BSA. Final pellets were resuspended in this medium to yield 80–90 mg protein/ml. Measurements were performed at 30 °C in KCl-based medium (120 mM KCl, 20 mM sucrose, 10 mM HEPES (pH adjusted to 7.3 with Trizma Base), 2 mM KH_2PO_4, and 2 mM $MgCl_2$), unless otherwise indicated. Other experimental details are given in figures and figure legends. The total mitochondrial protein was determined by the Biuret method using BSA as a standard [40].

2.3. Assessment of the intactness of mitochondrial membranes

The permeabilization of the IMM to ions and solutes causes the immediate swelling of mitochondria and the rupture of the outer mitochondrial membrane (OMM). The intactness of mitochondrial membranes was defined as the ratio of the rates of succinate-supported reduction of exogenous cytochrome *c* in the absence and in the presence of pore-forming peptide Alam (40 μg/mg protein) expressed in percent [29]. Mitochondria were used in experiments if contained less than 5% of damaged organelles (usually about 3%).

2.4. Recording of the permeabilization of mitochondrial membranes

The permeabilization of mitochondrial membranes for solutes was assessed by high-amplitude mitochondrial swelling. Mitochondrial swelling (a decrease in A_{540}) was recorded using a plate reader (Infinite 200 Tecan, Austria) and 96-well plates. Other details are given in figures and figure legends.

2.5. Assessment of ROS production

2.5.1. Hydrogen peroxide

The level of hydrogen peroxide in a mitochondrial suspension was measured in standard KCl-BM supplemented with 500 μM EGTA, 20 μM Amplex Red, and horseradish peroxidase (HRP) (3 U/ml). Resorufin accumulation was traced using a plate fluorimeter (Infinite 200 Tecan) in 96-well plates at excitation and emission wavelengths of 530 and 595 nm. For the quantitative assessment of hydrogen peroxide, fluorescence was calibrated by an excess of hydrogen peroxide at the end of measurements. In order to avoid light-induced resorufin formation, the fluorescence was measured once or two times a minute.

2.5.2. SA

The rate of SA production was assessed using the highly sensitive chemiluminescent probe MCLA [32]. The kinetics of MCLA-derived chemiluminescence (MDCL) was recorded using a plate reader (Infinite 200 Tecan). Each value on the curve is the mean ± S.E.M. of three integrations of luminescence for 900 ms expressed in arbitrary units.

2.5.3. Aconitase assay

Aconitase activity was measured by fluorescent monitoring of NADP reduction (Ex360/Em465) in a freshly prepared reaction mixture (50 mM Tris-HCl (pH 7.4), 5 mM sodium citrate, 0.6 mM $MnCl_2$, 200 μM NADP, and pig heart isocitrate dehydrogenase (1 unit/ml)) [41] with addition of Alam (20 μg/ml) for the permeabilization of the IMM. The reaction was started by the addition of aliquots (10 μg protein) of MCLA-free samples to 100 μl of assay mixture preequilibrated at 25 °C. The activity of aconitase was expressed in nmol min^{-1} mg $protein^{-1}$. Other experimental details are given in figure legends.

2.6. Generation of SA by potassium peroxide

Measurements were performed at 25 °C in KCl-based medium supplemented with 50 mM HEPES (pH adjusted to 7.3 with Trizma Base),

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