Inhibition of pro-oxidant-induced mitochondrial pyridine nucleotide hydrolysis and calcium release by 4-hydroxynonenal

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Intra- and extra-mitochondrial Ca^{2+} participates in vital cellular processes. This work investigates the influence of 4-hydroxynonenal (HNE) on pro-oxidant-induced Ca^{2+} release from rat liver mitochondria. Ca^{2+} movements across the mitochondrial inner membrane, the pyridine nucleotide redox state and pyridine (nicotinamide) nucleotide hydrolysis were analysed. HNE did not influence Ca^{2+} uptake by mitochondria, but inhibited in a concentration-dependent manner Ca^{2+} release induced by t-butylhydroperoxide (tbh). Total inhibition was achieved with about 50 μM -HNE. Ca^{2+} release induced by the pro-oxidant alloxan was also inhibited by HNE. Oxidation of pyridine nucleotides, induced by tbh through the concerted action of glutathione peroxidase, glutathione reductase and the energy-linked transhydrogenase, was not affected by up to 50 μM -HNE. In contrast, HNE inhibited pyridine nucleotide hydrolysis in a concentration-dependent manner. The data suggest that HNE toxicity may be in part attributed to an impaired intramitochondrial Ca^{2+} homeostasis.

INTRODUCTION

Intracellular Ca^{2+} is of central importance for many vital cellular processes such as cell growth and division, signal transduction, movement, secretion and short-term enzyme regulation [1]. To allow Ca^{2+} to serve these regulatory functions, fine tuning of its concentration is required. The cytosolic Ca^{2+} concentration in resting cells is kept between 0.1 and 0.2 μM , compared with millimolar concentrations in extracellular fluids. The intracellular Ca^{2+} concentration is modulated by binding of the ion to non-membranous proteins, and by the operation of membrane-bound Ca^{2+} transport systems. The existence of the latter has been established in the plasma membrane, the endoplasmic (sarcoplasmic) reticulum and mitochondria. The transport systems differ in Ca^{2+} affinity and transport capacity, and probably also in importance for the maintenance and development of physiological and pathological states of the cell [2]. Due to their high Ca^{2+} storage capacity, combined with a relatively low Ca^{2+} affinity, mitochondria act as safety devices against toxic increases of cytosolic Ca^{2+} [3].

Ca^{2+} enters and leaves energized mitochondria by separate routes. It is taken up electrophoretically in response to the mitochondrial membrane potential (negative inside) via a site that can be inhibited, e.g. by Ruthenium Red. Release of Ca^{2+} is electroneutral and, as shown mainly by the use of various pro-oxidants, most likely regulated by protein mono(ADP-ribosylation) (reviewed in [3]). However, this regulatory mechanism appears to operate even without added pro-oxidants, as recently shown with the ADP-ribose trap *m*-iodobenzylguanidine [4].

Highly reactive substances are formed during the oxidative breakdown of polyunsaturated fatty acid residues of membrane lipids. A major breakdown product is 4-hydroxynonenal (HNE), a compound with cytotoxic, hepatotoxic, mutagenic, genotoxic and other (e.g. chemotactic) properties (for a recent review, see [5]). We now report that HNE prevents Ca^{2+} release from mitochondria by inhibiting the intramitochondrial hydrolysis of NAD^+ to ADP-ribose and nicotinamide, and discuss possible

consequences of his inhibition for cellular Ca^{2+} homeostasis as related to the cytotoxicity of HNE.

METHODS

Isolation of mitochondria

Female Wistar rats (180–250 g) were starved overnight and killed by decapitation. Liver mitochondria were isolated by a conventional differential centrifugation method using a buffer containing 210 mM-mannitol, 70 mM-sucrose and 5 mM-Hepes, pH 7.4 (MSH buffer) plus 1 mM-EDTA as isolation medium. Mitochondria were washed twice in MSH buffer. The protein content of the final mitochondrial suspension was determined by the biuret method with bovine serum albumin as standard.

Labelling of mitochondrial pyridine nucleotides *in vitro*

Overnight-starved rats were injected intravenously with [*carboxy*- ^{14}C]nicotinic acid (12.5 μCi , 0.223 μmol). After 3 h, the animals were killed and liver mitochondria were isolated [6].

Standard incubation procedure

Mitochondria (2 mg of protein/ml) were incubated at 25 °C in 3 ml of MSH buffer with constant stirring. When appropriate, HNE was added at the indicated concentrations at the beginning of the incubation. A fine jet of O_2 was blown on to the surface of the suspension to prevent anaerobiosis. Reduction of mitochondrial pyridine nucleotides and release of endogenous Ca^{2+} was induced by 5 μM -rotenone. Mitochondria were then energized with 2.5 mM-potassium succinate. Ca^{2+} (60 nmol/mg of protein) was added and Ca^{2+} uptake was allowed to proceed for 2 min. Finally, t-butylhydroperoxide (tbh) or alloxan was added (time zero), preceded, when appropriate, by 2 nmol of Ruthenium Red/mg of protein.

Determination of Ca^{2+} uptake and release by mitochondria

Ca^{2+} movements across the mitochondrial inner membrane were monitored by dual-wavelength spectrophotometry in MSH buffer containing 50 μM -arsenazo III [7].

Abbreviations used: HNE, 4-hydroxynonenal; MSH buffer, 210 mM-mannitol, 70 mM-sucrose, 5 mM-Hepes, pH 7.4; tbh, t-butylhydroperoxide.

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Fig. 1. Pro-oxidant-induced release of Ca^{2+} from rat liver mitochondria

In the presence of arsenazo III, mitochondria were loaded with 60 nmol of Ca^{2+} /mg of protein. (a) At the arrow, Ca^{2+} release was initiated by the addition of 100 μM -tbh. (b) At the left arrow, 2 nmol of Ruthenium Red (RR)/mg of protein was added, followed by 100 μM -tbh (right arrow) (c) At the arrow, Ca^{2+} release was initiated by the addition of 2 mM-alloxan. Ca^{2+} movements across the mitochondrial inner membrane were monitored spectrophotometrically at 685–675 nm. Numbers next to the traces indicate HNE concentrations (μM). The results are from one experiment typical of eight.

Determination of mitochondrial pyridine nucleotides

Mitochondrial pyridine nucleotides were determined spectrophotometrically in an Aminco DW 2A spectrophotometer at 340–370 nm.

Determination of nicotinamide release

Pyridine nucleotides were labelled *in vivo* at the nicotinamide moiety (see above). Mitochondria isolated from the livers of these animals were incubated according to the standard procedure, and at zero time tbh was added. Release of nicotinamide was monitored by determining the remaining intramitochondrial radioactivity. To this end, 150 μl aliquots were withdrawn, filtered through Millipore filters (0.45 μm pore size) and rinsed twice with 150 μl of cold MSH buffer. The radioactivity remaining on the filters was measured in a liquid scintillation counter.

RESULTS

Fig. 1 shows the uptake of Ca^{2+} by energized rat liver mitochondria followed by pro-oxidant-induced Ca^{2+} release. HNE did not affect Ca^{2+} uptake. However, the tbh-induced release, which requires the concerted operation of glutathione peroxidase (EC 1.11.1.9), glutathione reductase [NAD(P)H: oxidized-glutathione oxidoreductase; EC 1.6.4.2] and the energy-linked transhydrogenase [NAD(P)⁺ transhydrogenase; EC 1.6.1.1] in mitochondria [3], was very sensitive to micromolar concentrations of HNE (Fig. 1a). When Ruthenium Red was added to mitochondria after completion of Ca^{2+} uptake, i.e. when re-uptake of any released Ca^{2+} (Ca^{2+} cycling [8]) was prevented, net Ca^{2+} release was observed even without addition of tbh [9]. This spontaneous release was to some extent prevented by HNE (results not shown). In accordance with previous findings [9] tbh also caused in the presence of Ruthenium Red, a stimulation of the Ca^{2+} -release pathway (Fig. 1b). The tbh-stimulated release was also sensitive to HNE.

Like tbh, the pro-oxidant alloxan induced net Ca^{2+} release from intact mitochondria by activation of the specific pyridine-nucleotide-dependent release pathway. However, alloxan does not engage the glutathione peroxidase/transhydrogenase enzyme cascade. Rather, this pro-oxidant oxidizes intramitochondrial pyridine nucleotides in a predominantly non-enzymic fashion [7]. Fig. 1(c) shows that 50 μM -HNE retarded Ca^{2+} release induced by 2 mM-alloxan.

Pyridine nucleotide oxidation and hydrolysis is a prerequisite for the pro-oxidant-induced mitochondrial Ca^{2+} release, with hydrolysis requiring intramitochondrial Ca^{2+} [3]. Intramito-

chondrial pyridine nucleotides *in vitro* can become extensively and irreversibly oxidized due to Ca^{2+} -dependent enzymic splitting of NAD^+ to ADP-ribose and nicotinamide [3,10]. Since tbh and alloxan cause pyridine nucleotide oxidation by different pathways (see above), the inhibition of Ca^{2+} release induced by either of these compounds suggested that HNE does not interfere with pro-oxidant-induced pyridine nucleotide oxidation. Indeed, HNE up to 50 μM clearly did not slow down the initial rate of oxidation induced by tbh (Fig. 2). HNE does, however, allow partial or nearly complete re-reduction of pyridine nucleotides. With 100 μM -HNE, the rate and steady state of pyridine nucleotide oxidation was clearly diminished and the onset of re-reduction was retarded, possibly due to limited alkylation of glutathione.

The re-reduction of intramitochondrial pyridine nucleotides in the presence but not in the absence of 20 μM or more HNE shown in Fig. 2 strongly suggested that HNE inhibited the pro-oxidant-induced Ca^{2+} release by preventing pyridine nucleotide hydrolysis. To obtain unequivocal proof of this, we measured release of nicotinamide from mitochondria as an indicator of intramitochondrial pyridine nucleotide hydrolysis [6]. Fig. 3 shows that HNE is indeed a potent inhibitor of this reaction.

DISCUSSION

There is convincing evidence that protein mono(ADP-ribosylation) regulates Ca^{2+} release from intact respiring mito-

Fig. 2. Changes in the redox level of mitochondrial pyridine nucleotides

The redox level of mitochondrial pyridine nucleotides was monitored spectrophotometrically at 340–370 nm. Mitochondria were energized with succinate and loaded with 60 nmol of Ca^{2+} /mg of protein. At the arrow 100 μM -tbh was added. Numbers next to the traces indicate HNE concentrations (μM). The results are from one experiment typical of five.

Fig. 3. Hydrolysis of pyridine nucleotides in rat liver mitochondria

Intramitochondrial pyridine nucleotides were labelled *in vivo* at the nicotinamide moiety. Incubation was according to the standard procedure with and without HNE. Mitochondria were loaded with 60 nmol of Ca^{2+} /mg of protein. At zero time, 100 μM -tbh was added, and 10 min later the release of nicotinamide from mitochondria was measured by Millipore filtration. The results are from one experiment typical of three.

chondria [2,11]. Release requires both oxidation of pyridine nucleotides and their hydrolysis to ADP-ribose and nicotinamide [2], and is accompanied by a transient modification of intramitochondrial proteins with mono(ADP-ribose) [11]. As reported here, HNE inhibits the pro-oxidant-induced as well as the spontaneous Ca^{2+} release from mitochondria by preventing the key reaction during protein ADP-ribosylation, i.e. pyridine nucleotide hydrolysis. The inhibition of hydrolysis is indicated by the reversibility of pyridine nucleotide oxidation [10] and by the analysis of nicotinamide release from mitochondria [6]. The action of HNE is, therefore, comparable with that of ATP or cyclosporine A, which also inhibit pyridine nucleotide hydrolysis [12,13], but is different from that of the other known inhibitor of the pro-oxidant-induced Ca^{2+} release, *m*-iodobenzylguanidine, which inhibits presumably by competing with the natural acceptor proteins of ADP-ribose [4].

Cyclosporine A has previously been shown by the groups of Crompton [14] and Pfeiffer [15] to inhibit the hydroperoxide-induced Ca^{2+} release from heart mitochondria and swelling of liver mitochondria respectively. The inhibition of Ca^{2+} release by such diverse compounds as HNE, ATP, cyclosporine A and *m*-iodobenzylguanidine provides further clear evidence that release is not due to damage of mitochondria, a fact recently also acknowledged by Pfeiffer and coworkers [15].

The inhibition of mitochondrial respiration and electron flow *in vitro* by millimolar 4-hydroxypentenol and HNE concentrations has previously been reported by others [16,17]. That such inhibition also occurs *in vivo* is not likely, since the average HNE

concentration in normal rat liver is about 0.5–3 μM and increases to about 5 μM in vitamin E deficiency [18]. In mitochondria, the most abundant polyunsaturated fatty acids belong to the *n*–6 series, and the *n*–6-derived HNE is probably present in these organelles in somewhat higher than average amounts. The significant inhibition of Ca^{2+} release from mitochondria by as little as 10 μM -HNE in the present study *in vitro* therefore suggests that inhibition also occurs *in vivo*, particularly during enhanced lipid peroxidation in mitochondria.

The cytotoxicity of HNE may be partly due to inhibition of mitochondrial Ca^{2+} release. Since several mitochondrial dehydrogenases are regulated by micromolar concentrations of Ca^{2+} [19], long-term overloading of mitochondria with Ca^{2+} due to a deactivated release pathway may severely compromise these enzymes. In addition, ATP [20] and RNA [21] synthesis are inhibited by increased amounts of mitochondrial Ca^{2+} . HNE may, therefore, be cytotoxic by indirectly shutting off the mitochondrial energy supply.

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