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The Effect of the Lipid Peroxidation Product 4-Hydroxynonenal and of its Metabolite 4-Hydroxynonenoic Acid on Respiration of Rat Kidney Cortex Mitochondria

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In rat kidney cortex mitochondria, 4-hydroxynonenal inhibits state 3 respiration as well as uncoupled respiration at micromolar concentrations. The inhibition is more distinct for NAD-linked than for FAD-linked respiration. 4-Hydroxynonenal increases the state 4 respiration. It is assumed that 4-hydroxynonenal behaves like a decoupling agent. 4-Hydroxynonenal augments the inhibitory effect of 2,4-dinitrophenol observed at superoptimal concentrations. 4-Hydroxynonenal is metabolised by renal mitochondria, and 4-hydroxynonenoic acid is one of the metabolites generated. This metabolite is without effect on respiration at concentrations up to 50 μM. Therefore, the effect of 4-hydroxynonenal on respiration is not mediated by this fatty acid derivative formed during respiratory measurements.

Key words: 4-hydroxynonenal, 4-hydroxynonenoic acid, oxidative phosphorylation, kidney mitochondria

Abbreviations: DNP, 2,4-dinitrophenol; FFA, free fatty acids; HNA, 4-hydroxy-2,3-trans-nonenoic acid (4-hydroxynonenoic acid); HNE, 4-hydroxy-2,3-trans-nonenal (4-hydroxynonenal); RCR, respiratory control ratio

INTRODUCTION

It is well-established that changes in mitochondrial function during ischemia are crucial in cell survival. Mechanisms contributing to ischemic dysfunction of mitochondria are widely investigated.[1,2] One mechanism comprises ischemic changes of phospholipids and impairing interactions between their degradation products and mitochondria.[3,4]

Membrane phospholipid breakdown with accumulation of fatty acids, diacylglycerol, and lysophospholipids as well as lipid peroxidation has been observed in brain, heart, liver and kidney after ischemia.[5–8] Lipid peroxidation is associated with the formation of aldehydes. 4-Hydroxy-2,3-trans-nonenal (4-hydroxynonenal, HNE) is a major product of lipid peroxidation formed by the

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degradation of omega-6-polyunsaturated fatty acids, e.g. arachidonic acid.[9]

HNE is a compound with genotoxic, cytotoxic, mutagenic and chemotactic activity and produces a multitude of effects such as depletion in glutathione, disturbance of calcium homeostasis, inhibition of DNA, RNA and protein synthesis, initiation of lipid peroxidation, and inhibition or activation of some specific enzymes. The mechanisms by which HNE damages cells in a manner leading to cell death are not clear.[10]

Recently, it has been described that a significant inhibition of prooxidant-induced mitochondrial pyridine nucleotide hydrolysis and Ca^{2+} release by as little as 10 μM HNE occurs *in vitro* and may be important *in vivo* for cytotoxicity.[11] Aldehydes like formaldehyde,[12] acetaldehyde,[13] maleylaldehyde[14] and 4-hydroxypentenal[15] inhibit respiration. Generally, the toxicity of aldehydes strongly increased with increasing lipophilicity,[12] thus HNE might be a very potent compound in inhibiting mitochondrial functions. However, the inhibition of respiration as a possible mechanism of HNE-induced cell death has not been studied systematically. In two undetailed reports with liver mitochondria, an inhibition of respiration by HNE has been shown.[14,16]

HNE formed from accumulating polyunsaturated fatty acids, especially arachidonic acid,[12] might contribute to the ischemic impairment of oxidative phosphorylation. Thus, the in vitro effect of HNE on respiration of renal cortical mitochondria is investigated in this study. This paper shows that micromolar HNE concentrations impair respiration of kidney mitochondria.

MATERIALS AND METHODS

Materials

ADP, aldehyde dehydrogenase (EC 1.2.1.5.) and NAD were obtained from Boehringer, Mannheim, Germany. 4-Hydroxynonenal was a gift from H. Esterbauer, Graz, Austria. Other chemicals used were of analytical grade.

Isolation of mitochondria

Male Wistar rats (Schönwalde, Germany) weighing 250–300 g were used. Rat kidney cortex mitochondria were isolated by a standard procedure.[17] The mitochondrial fraction was washed two times and finally resuspended in a medium consisting of 210 mM mannitol, 70 mM sucrose and 1 mM EDTA adjusted to pH 7.4 with 1 mM Tris.

Determination of respiratory parameters

Respiratory rates were measured at 25°C with a Clark-type electrode (Metra, Radebeul, Germany) using a medium of 210 mM sucrose, 10 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 60 mM Tris-HCl, pH 7.4 and either 10 mM succinate or both 10 mM glutamate and 10 mM malate.[17] About 1 mg/ml mitochondria were preincubated with HNE (bolus addition of HNE*) for 1 min before substrates were added. 30 sec after that, state 3 respiration was induced by 250 μM ADP. That means the measurement of mitochondrial respiration parameters was carried out 1.5 min after bolus addition of HNE. State 4 respiration and uncoupled respiration were measured after depletion of ADP and stimulation by 50 μM 2,4-dinitrophenol (DNP), respectively.

Preparation of 4-hydroxynonenoic acid

Desired HNE concentrations were preincubated in the medium used for respiratory measurements which contains additionally 2 U/ml aldehyde dehydrogenase and 0.4 mM NAD. HNE is oxidized to the corresponding 4-hydroxy-2,3-trans-

*The use of high bolus concentration for imitation of pathophysiologically relevant HNE concentration during the measurement is necessary because of rapid degradation of HNE in mitochondria (Ullrich *et al.* FEBS Lett. 352 (1994) 84–86). The initial bolus concentration should be high to reach physiologically relevant concentrations in the mitochondria. After 1.5 min (the beginning of the measurement of respiration) only 13% of HNE remain in the media.

nonenoic acid (4-hydroxynonenoic acid, HNA) by preincubation over 60 minutes as checked by measuring NADH absorbance at 340 nm or by HNE and HNA concentration, respectively, applying the following HPLC method. After completion of the HNA formation, respiratory rates were determined as described above. Controls were performed without aldehyde dehydrogenase but with NAD.

HNE and HNA determination by HPLC

HNE and HNA extracted with acetonitrile:acetic acid (94:6, v/v) were determined by HPLC separation using a 5 μM RP 18 column (Supelco, Bellefonte, PA, USA; 150 × 4.6 mm I.D.), an acetonitrile:water eluent (40:60, v/v), a flow rate of 1 ml/min and detection at 223 nm.

Other determinations

The protein content was determined by a biuret method after precipitation with trichloroacetic acid using human serum albumin as standard.[17]

RESULTS

Inhibition of respiration by HNE

HNE decreases the state 3 respiration rate of renal mitochondria using succinate as substrate (Figure 1a). At 500 μM HNE, the highest concentration applied, the state 3 respiration rate is diminished to about 70% of the control. The uncoupled respiration is decreased progressively by HNE after passing through a flat maximum at about 125 μM HNE. The state 4 respiration is raised with the HNE concentration. Compared with the control, the state 4 respiration is increased about 2-fold at 500 μM HNE. Whereas the RCR is markedly decreased by HNE the ADP/O ratio is only slightly influenced (Figure 1b).

Both the state 3 and uncoupled respiration supported by glutamate/malate are progressively diminished by HNE (Figure 2a). The HNE-induced decline of uncoupled respiration proceeds stronger than that of state 3 respiration. The state 4 respiration is increased by HNE. The RCR is diminished more pronounced by raising HNE

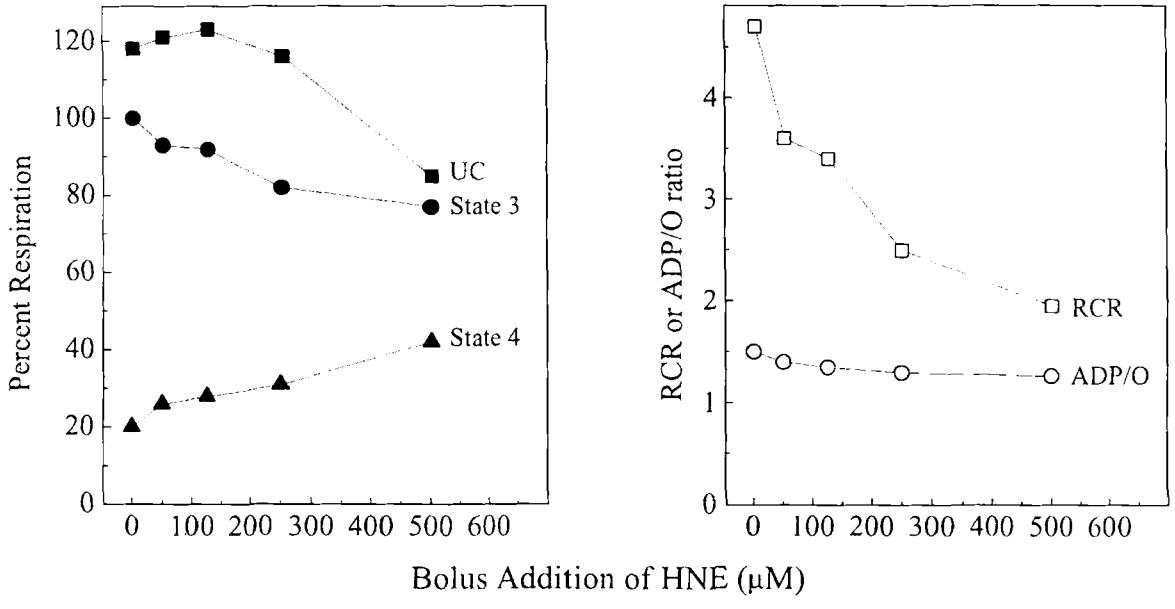

FIGURE 1 Succinate-supported respiration as function of 4-hydroxynonenal concentration. Values are given as percentage of state 3 respiration (282 ± 18 nAtom O/min/mg protein) without addition of 4-hydroxynonenal. Values in both panels are means of 4 experiments, for which S.E.'s were less than 7%. (UC – uncoupled respiration)

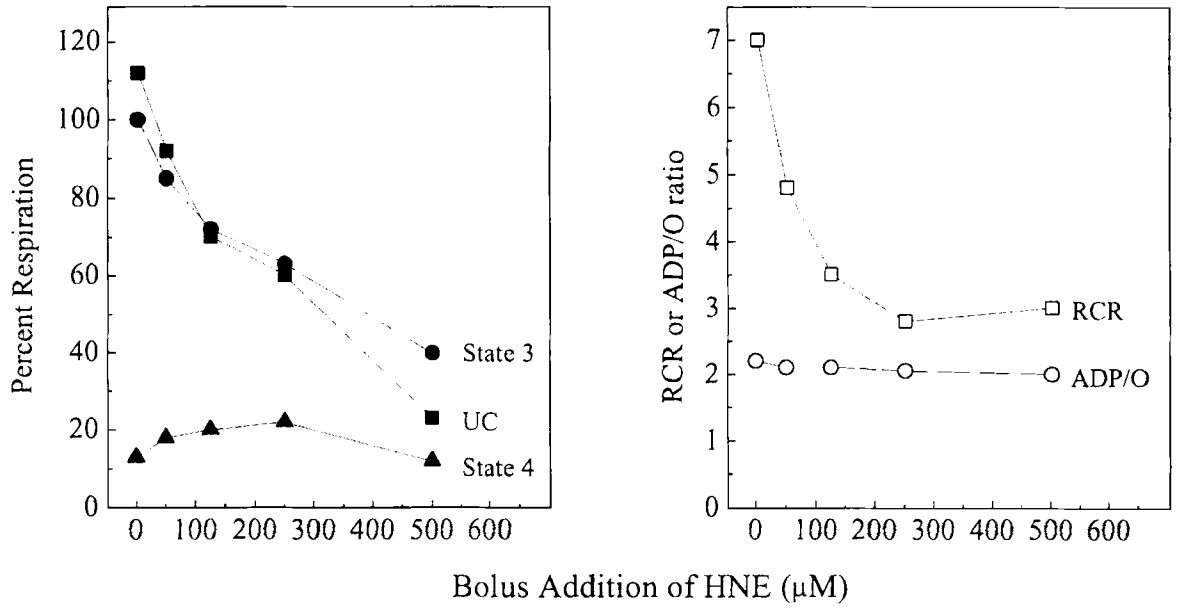

FIGURE 2 Glutamate/malate-supported respiration as function of 4-hydroxynonenal concentration. Values are given as percentage of state 3 respiration (139 ± 18 nAtom O/min/mg protein) without addition of 4-hydroxynonenal. Values reported are the means of 4 experiments with S.E.'s always less than 10%. n = 4. (UC – uncoupled respiration)

concentrations than the ADP/O ratio (Figure 2b). HNE is more effective on the NAD-linked respiration than on the FAD-linked. For example, the I_{50}-value of HNE in decreasing RCR amounts to about 250 μM or 125 μM for the FAD-linked or NAD-linked respiration, respectively.

As is well known from the literature, DNP stimulates the respiration at low concentrations and inhibits it at higher ones.[18] Figure 3 demonstrates the effect of HNE on the DNP titration of succinate-supported state 3 respiration. Increasing HNE concentrations shifts the optimal stimulating DNP concentration to lower values.

Formation and effect of 4-Hydroxynonenoic acid

HNE is degraded by mitochondria. The HNE degradation is connected with the formation of HNA as detected by HPLC analysis. After incubation for 10 min about 8 μM HNA are produced (Table 1). That means that less than 10% of the total HNE was converted to HNA, one of the possible HNE metabolites.

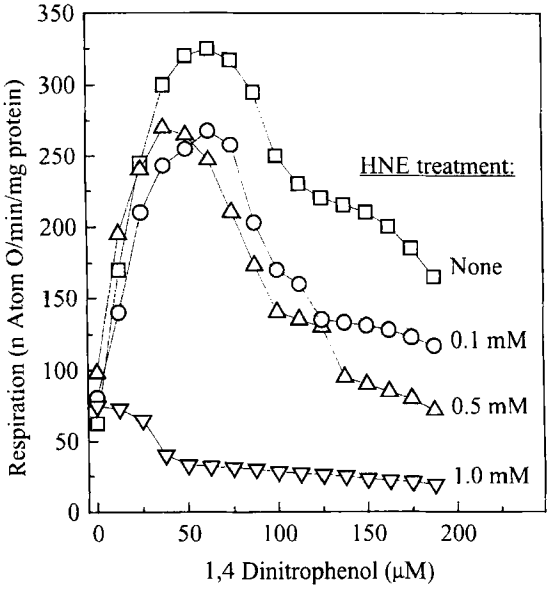

FIGURE 3 Influence of 4-hydroxynonenal on succinate-supported respiration as function of 2,4-dinitrophenol concentration. All values are means of 4 independent experiments, for with S.E.'s were always less than 10%.

TABLE 1 Formation of 4-hydroxynonenoic acid from 100 μM HNE in mitochondrial suspension.

time (min)	HNA (μM)[1)]	HNE (μM)	HNA formed (%)
0	0	100	0
1	1.44 ± 0.5	23.6 ± 3.2	1.9
10	6.69 ± 0.8	8.1 ± 3.2	7.2

[1)]Values are given as mean ± S.D., n = 3.

Fatty acids are well-known inhibitors of mitochondrial respiration.[19] Therefore, the influence of the fatty acid derivative HNA on respiration was investigated. HNA prepared by preincubation of HNE with aldehyde dehydrogenase has no effect on state 3, uncoupled respiration or the ADP/O-ratio of renal mitochondria (Table 2). The state 4 respiration is increased and the RCR decreased by HNA. This uncoupling effect is more pronounced with glutamate/malate than with succinate.

DISCUSSION

HNE effects on mitochondria have been studied, since its precursors, especially arachidonic acid, accumulate in several organs at ischemia.[5–8] It is shown that HNE impairs the respiration of rat kidney cortex mitochondria. The pattern of respiratory parameters observed with HNE is very similar to that described with free fatty acids (FFA). Thus, HNE and FFA[3,19] stimulate state 4 respiration. The stimulating effect of FFA is attributed to both decoupling[20] and uncoupling[21] of oxidative phosphorylation by intrinsic slip induction or protonophore shuttling, respectively. From experiments published recently it has been concluded that FFA acts as protonophore via the ADP/ATP-carrier.[22] Because HNE has no protonophoric properties the stimulation of state 4 respiration might be caused by intrinsic slip induction described, e.g. with general anesthetics.[23] Nevertheless, further experiments are required to define HNE as a decoupling agent by criteria summarized recently.[19] As shown by HPLC analysis, renal mitochondria oxidise HNE to HNA. FFA affects respiration at μmolar concentrations.[3,19] Therefore, it might be assumed that HNA formed during measurements of respiration is the true inhibiting compound. Respiratory measurements performed with this fatty acid derivative prepared from HNE with aldehyde dehydrogenase exclude this possibility. The slow uncoupling

TABLE 2 Effect of different concentrations of 4-hydroxynonenoic acid on respiration of renal mitochondria.

HNA (μM)	state 3[1)]	state 4 relative values[2)]	uncoupled	RCR	ADP/O
			succinate		
0	100 ± 0	26.5 ± 2.3	109.8 ± 7.1	3.80 ± 0.35	1.72 ± 0.08
5	95.6 ± 6.5	28.4 ± 5.9	110.1 ± 8.9	3.45 ± 0.84	1.60 ± 0.14
10	91.2 ± 1.1	28.3 ± 6.9	103.9 ± 10.6	3.33 ± 0.69	1.58 ± 0.06
20	89.2 ± 4.9	29.2 ± 8.6	104.8 ± 10.3	3.04 ± 0.92	1.68 ± 0.25
50	90.7 ± 4.8	31.5 ± 6.5	106.3 ± 9.4	2.96 ± 0.76	1.56 ± 0.12
			glutamate/malate		
0	100 ± 0	14.6 ± 4.6	103.4 ± 13.4	6.01 ± 0.53	2.37 ± 0.05
5	96.9 ± 8.7	23.4 ± 6.5	115.2 ± 12.	4.31 ± 0.90	2.20 ± 0.06
10	99.5 ± 9.6	25.8 ± 3.5	110.7 ± 6.2	3.99 ± 0.66	2.23 ± 0.14
20	92.6 ± 9.9	25.0 ± 5.2	118.2 ± 6.6	3.55 ± 0.52	2.25 ± 0.08
50	97.2 ± 5.9	25.1 ± 5.7	111.8 ± 11.2	3.89 ± 0.94	2.36 ± 0.09

[1)]state 3, state 4, uncoupled, RCR, ADP/O–state 3 respiration, state 4 respiration, uncoupled respiration, respiratory control ratio, ADP/O ratio

[2)]Values are given as % of state 3 respiration without addition of 4-hydroxynonenoic acid; mean ± S.D., n = 3.

observed with HNA, especially on NAD-linked respiration, corresponds to the effect known with FFA.

HNE decreases the rate of state 3 respiration. The ADP/ATP-carrier has a considerable share in flux control of the respiration.[24] The activity of the ADP/ATP-carrier is decreased in ischemic organs.[2,25] Ischemia decreases the content of the ADP/ATP-carrier in renal mitochondria. It is supposed that the ADP/ATP-carrier is inactivated by oxidative reactions.[26] Recently, it has been found that peroxidative damage to cardiac mitochondria, induced with *t*-butylhydroperoxide and $CuCl_2$, generates a modified ADP/ATP-carrier. The modification may be due to covalent adduct formation of the ADP/ATP-carrier protein with peroxidised cardiolipin.[27] It is difficult to decide whether the ADP/ATP-carrier activity is diminished by HNE, but since the rate of uncoupled respiration, generally accepted to express the maximal respiratory capacity, is also depressed, it is concluded that respiratory chain proteins are inhibited by HNE. HNE is more effective on NAD-linked than on FAD-linked respiration. This finding is in accordance with data published with 4-hydroxypentenal. Complex I of the respiratory chain is assumed to be more sensitive than complex II against this aldehyde.[15]

DNP inhibits the respiratory rate at higher concentrations.[18] HNE enhances the inhibitory effect of superoptimal DNP concentration on the respiratory chain. This effect is also observed with 4-hydroxypentenal.[15] The synergistic effects of HNE and DNP cause the observed stronger HNE-induced decline of uncoupled respiration compared with that of NAD-linked state 3 respiration. Using phenolic compounds the cytochrome bc1 of complex III has been identified as the inhibitory site of these uncouplers.[28] HNE seems to interact with complex III, too.

The ischemia-induced dysfunction of renal mitochondria is characterized by a diminished rate of both state 3 respiration and uncoupled respiration.[29] At ischemia both complexes I and III are injured.[30] Mitochondria generate superoxide radicals, especially at complexes I and III, and consecutively hydrogen peroxide.[31] Conditions connected with a highly reduced state of the respiratory chain, e.g. at ischemia or at state 4 respiration, are predestined for mitochondrial radical generation.[32] Among the mechanisms shown to be involved in mitochondrial injury[2,25,26,30] the radical-induced impairment of the respiratory chain is discussed.[31,32] Nohl *et al.*[33] have suggested that mitochondria are likely to play a pathogenetic role in the reperfusion injury of heart, both by an impairment of energy conservation and by their transition to a potent $O_2^{\bullet -}$ radical generator. With heart mitochondria, there is evidence that an exogenous NADH-dehydrogenase is mainly responsible for functional changes of these organelles during ischemia/reperfusion.[33]

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