Hydroperoxy Fatty Acid Cycling Mediated by Mitochondrial Uncoupling Protein UCP2*

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Functional activation of mitochondrial uncoupling protein-2 (UCP2) is proposed to decrease reactive oxygen species production. Skulachev and Goglia (Skulachev, V. P., and Goglia, F. (2003) FASEB J. 17, 1585–1591) hypothesized that hydroperoxy fatty acid anions are translocated by UCPs but cannot flip-flop across the membrane. We found that the second aspect is otherwise; the addition of synthesized linoleic acid hydroperoxides (LAAOOH, a mix of four isomers) caused a fast flip-flop-dependent acidification of liposomes incompatible with the linoleic acid (LA)-dependent acidification. Using *Escherichia coli*-expressed UCP2 reconstituted into liposomes we found that LAAOOH induced purine nucleotide-sensitive H+ uniport in UCP2-proteoliposomes with higher affinity than LA (Km values 97 μM for LAAOOH and 275 μM for LA). In UCP2-proteoliposomes LAAOOH also induced purine nucleotide-sensitive K+ influx balanced by anionic charge transfer, indicating that LAAOOH was also transported as an anion with higher affinity than linolate anion, the Km values being 90 and 350 μM, respectively. These data suggest that hydroperoxy fatty acids are transported via UCP2 by a fatty acid cycling mechanism. This may alternatively explain the observed activation of UCP2 by the externally generated superoxide. The ability of LAAOOH to induce UCP2-mediated H+ uniport points to the essential role of superoxide reaction products, such as hydroperoxy radical, hydroxyl radical, or peroxynitrite, initiating liperoxidation, the released products of which support the UCP2-mediated uncoupling and promote the feedback down-regulation of mitochondrial reactive oxygen species production.

Mitochondria produce a substantial amount of superoxide anion (O2⋅−) (1–11) at Complex I (by a yet unresolved mechanism, O2⋅− is released to the matrix) (2) and via autooxidation of the ubisemiquinone anion radical at Complex III (1–11), where O2⋅− could be released into both sides of the membrane (2). A spectrum of radical and non-radical compounds is produced from O2⋅−, which are commonly called reactive oxygen species (ROS).1 These include radicals (O2⋅−, hydroperoxyl (HO2⋅), hydroxyl (OH⋅), peroxyl (RO2⋅), alkoxyl (RO⋅)) and nonradical species such as H2O2 and singlet oxygen (3, 4). The list is extended by peroxyxenonite, which is formed by the reaction of O2⋅− with nitric oxide, NO (12, 13). Most of the O2⋅− is converted to H2O2 by matrix manganese-superoxide dismutase (14) and by cytosolic CuZn-superoxide dismutase, which also exists in the mitochondrial intramembrane space (2). H2O2, which has been recognized as an important signaling molecule (15–17), is then degraded by several redox systems, including glutathione peroxidases, which reduce H2O2 to water (3–7). At pH 6.8, ~1% of O2⋅− is protonated to highly reactive species, hydroperoxyl radical HO2⋅ (pKa 4.8) (4). H2O2 may be converted into highly reactive ‘OH by a reaction with transition metals, namely Fe3+ (Fenton reaction) (3, 4).

The ‘OH is an extremely reactive oxidizing radical that will react to most biomolecules at diffusion-controlled rates. The hydroxyl radical can also oxidize nitrate to nitrogen dioxide (NO2) and react with bicarbonate yielding the very reactive carbonate radical anion under physiological conditions (4). All of these highly reactive radicals (HO2⋅, ‘OH, and CO3⋅−) attack proteins and DNA, but, in addition, a substantial liperoxidation must occur in vivo, because polyunsaturated hydrocarbon chains are very susceptible to allelic hydrogen abstraction (3, 4, 18–20). Once carbon-centered lipid radicals (RC⋅) are generated, they react with O2 giving peroxyl radicals (ROO⋅), which react for example with the neighbor polyunsaturated hydrocarbon side chain yielding hydroperoxides (ROOH) and other RCR. The reaction is thus propagated by many cycles (18). Fatty acid hydroperoxides (FAOOH) can be cleaved off from the phospholipids by phospholipase A2 (PLA2) (20), e.g. by its mitochondrial Ca2+-dependent isoform induced by superoxide (21) or by the Ca2+-independent isoform. FAAOOH are transient, non-radical but reactive species, which are eventually degraded by glutathione peroxidase or phospholipid hydroperoxide glutathione peroxidase to the corresponding hydroxy fatty acids (FAOOH). FAAOOH may also decompose to toxic epoxy acids and α,β,γ,δ-unsaturated aldehydes (18).

It has been found that even a slight increase of H+ backflow into the matrix may substantially suppress mitochondrial ROS formation (7–11). The physiological H+ backflow during ATP formation of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** The abbreviations used are: ROS, reactive oxygen species; FA(s), fatty acids(s); FAOH, hydroxy-fatty acid; FAAOOH or FAAOOH-COOH, fatty acid hydroperoxides; HNE, 4-hydroxy-2-nonenal; LA, linoleic acid; LAAOOH, linoleic acid hydroperoxides; PLA2, phospholipase A2; PUFA(s), polyunsaturated FAs; SPQ, 6-methoxy-N-(3-sulfopropyl)quinolinium; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; UCP2, “ubiquitous” uncoupling protein; UCP, uncoupling protein.

1 The abbreviations used are: ROS, reactive oxygen species; FA(s), fatty acid(s); FAOH, hydroxy-fatty acid; FAAOOH or FAAOOH-COOH, fatty acid hydroperoxides; HNE, 4-hydroxy-2-nonenal; LA, linoleic acid; LAAOOH, linoleic acid hydroperoxides; PLA2, phospholipase A2; PUFA(s), polyunsaturated FAs; SPQ, 6-methoxy-N-(3-sulfopropyl)quinolinium; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; UCP2, “ubiquitous” uncoupling protein; UCP, uncoupling protein.
synthesis also suppresses ROS formation (7–9); therefore most ROS are produced in the non-phosphorylating state. This effect of uncoupling or ATP synthesis is due to increased respiration and hence concomitantly shortened lifetime of UQ" and the lowered oxygen tension in the microenvironment which results in reduced rate of O\textsubscript{2} formation (7–11). These considerations led to the proposal that a physiological role of uncoupling protein-2 (UCP2), which is present in very low amounts in most tissues (22–32), is to down-regulate mitochondrial ROS production (25, 26). The ability to reduce ROS and/or liperoxidation not only locally in the mitochondria but within the cell or even in the extracellular space has also been ascribed to these novel UCPs (28, 29).

Brand and co-workers (33, 34) stated initially that superoxide itself “activates” UCP2 by an unspecified mechanism from the matrix side. This is an attractive proposal, because it provides a feedback mechanism for the control of mitochondrial ROS production. Recently, however, that group has abandoned the direct interaction of O\textsubscript{2} with UCP2 and instead ascribes this ability to end-products of the liperoxidation cascade, such as 4-hydroxy-2-nonenal (HNE) (35–37). They found that mitochondrially targeted α-phenyl-N-tert-butynitrite prevented UCP2 activation by externally produced O\textsubscript{2} but did not prevent activation by HNE, indicating that the HNE effect occurs downstream (36). Brand and co-workers (36) therefore predict a direct (covalent) interaction of ROS-derived activators with UCP2.

We suggest an alternative mechanism in which the FAOOH produced by elevated ROS behave as cycling substrates of the UCPs. In this mechanism, UCP acts as a flipase to transport the fatty acid (FA) anionic head group across the membrane. The FA is protonated and rapidly flip-flops back across the membrane, delivering protons electroneutrally across the membrane (38–46). Thus, FAs are not viewed as allosteric activators but rather as the transported substrates of any UCP. Skulachev and Goglia (47) have speculated that FAOOH-COO" may be the anionic transport substrates of the UCPs, but they predicted that the protonated FAOOH-COOH cannot diffuse through the membrane, which would prevent uncoupling. If that speculation were correct, FAOOH would behave like long-chain alkylsulfonates (38, 43) or so-called inactive FAs (48, 49). The strong acidic character of the former (38, 43) and a "U-shape" of the latter compounds in the membrane (48) prevent their flip-flop with protons across the bilayer.

We examined interactions of linoleic acid hydroperoxides (LAOOH) (50, 51) with liposomal membranes and with reconstituted UCP2. We found that LAOOH does rapidly flip-flop across the membrane, resulting in the delivery of protons to the interior. Moreover, LAOOH supports uncoupling in liposomes containing UCP2, and it does so with a higher affinity than was observed for linoleic acid itself. We concluded that LAOOH is an efficient cycling substrate of UCP2. The finding that FAOOH supports UCP-mediated uncoupling suggests an alternative explanation for the reported activation of UCP2 by externally generated superoxide observed exclusively in the presence of FAs (33). Our suggested mechanism may serve physiologically as a feedback regulation accelerating the suppression of mitochondrial ROS production.

**EXPERIMENTAL PROCEDURES**

Most of the chemicals were purchased from Sigma. LAOOH (Fig. 1) was synthesized in the São Paulo laboratory as described previously (50, 51). Hydroxylapatite, Bio-Gel HTP, and Bio-Beads SM2 were from Bio-Rad. Octylpentaoxyethylene was from Bachem Feinchemikalien, Bubendorf, Switzerland. Other materials for reconstitution were from the same sources as described elsewhere (32, 38–39, 43, 45, 46). All other chemicals were of a reagent grade.

**Results**

**Flip-Flop Acidification of Liposome Interior upon Addition of LAOOH**—To determine whether protonated fatty acid hydroperoxides are capable of crossing the lipid bilayer, we studied the interior acidification of liposomes caused by the addition of LAOOH. The addition of 100 μM linoleic acid causes a
reduced quenching of SPQ fluorescence, demonstrating acidification of liposomes by FA (Fig. 2A, trace a). The addition of 100 μM LAOOH caused an identical reduction in SPQ quenching (Fig. 2A, trace b), demonstrating the ability of LAOOH to cross the lipid bilayer in the protonated form. Fig. 2B shows that the extent of total H⁺ release depends on the amount of FA added for both linoleic acid (squares) and LAOOH (circles). Previous results showed that FAs yield ~2.5% of their protons to the intraliposomal compartment (48). For LAOOH and linoleic acid the observed yield was 2.2–2.5% at pH 7.2 (Fig. 2B), which is in good agreement with the previous results. We concluded that the ability of LAOOH to flip-flop across the lipid bilayer is similar to the flip-flop of linoleic acid and other natural FAs.

LAOOH Cycling Mediated by UCP2 Reconstituted in Liposomes—Electrophoretic H⁺ flux in proteoliposomes was initiated by adding valinomycin in the presence of an inward K⁺ gradient. LAOOH induced an H⁺ efflux (Fig. 3, trace a) that was inhibited up to 50% by 2.5 mM GDP added to the assay medium (Fig. 3, trace b). Similar inhibition was observed when 2.5 mM ATP was used (not shown). The results with LAOOH were similar to those obtained using the parent FA, linoleic acid (Fig. 3, trace c). In protein-free liposomes neither LAOOH nor linoleic acid induced substantial H⁺ efflux (Fig. 3, traces d and e). Moreover, this background H⁺ efflux in liposomes was similar to fluxes obtained when 2.5 mM ATP was present inside and outside of the UCP2-containing proteoliposomes (not shown). The lack of H⁺ efflux in the absence of UCP2 and the observed inhibition by purine nucleotides indicate the participation of UCP2. Although the extent of flip-flop acidification was equal for 100 μM LAOOH and 100 μM linoleic acid, LAOOH induced faster H⁺ efflux of a higher extent in UCP2-proteoliposomes (Fig. 3, trace a versus c).

Kinetics of LAOOH-COO⁻/LAOOH-COOH Cycling as Compared with Linolate/Linolate Acid Cycling—To examine the ability of LAOOH to cause faster UCP2-catalyzed H⁺ fluxes than linoleic acid, we varied the total concentration of added FA and evaluated kinetics of valinomycin-induced H⁺ efflux. The obtained rates were corrected for fluxes in the absence of UCP2, and the net fluxes were normalized to the protein content (Fig. 4). We found that the apparent affinity of UCP2 for LAOOH and LA, taken as the reciprocal $K_m$, from an Eadie-Hofstee plot (Fig. 4), is higher for the hydroperoxide mixture (LAOOH) than for LA. The apparent $K_m$ was 97 μM for LAOOH and 275 μM for linoleic acid, whereas the $V_{max}$ values were quite similar, 18.8 or 17.7 μmol/min/mg of protein$^{-1}$, respectively (Fig. 4). In a series of four experiments, the mean value (± S.D.) of $K_m$ for the LAOOH-induced H⁺ fluxes was $97 ± 18$ μM, and the $V_{max}$ was $-20$ μmol/min/mg of protein$^{-1}$, whereas the $K_m$ for linoleic acid was $290 ± 40$ μM. If we assume that the kinetics of FA-dependent UCP2-catalyzed H⁺ fluxes consist of the flip-flop of protonated FA and UCP-catalyzed uniport of FA anion, we conclude that because the flip-flop activities of LAOOH and linoleic acid are similar, the differences between $K_m$ values must originate in the interactions between UCP2 and FA.

Uniport of LAOOH-COO⁻ Anions and Linolate Mediated by the Reconstituted UCP2—To demonstrate that mitochondrial
uncoupling protein UCP2 is able to mediate uniport of anionic hydroperoxy derivatives of linoleic acid, we measured the charge transfer in proteoliposomes containing UCP2 by measuring the concomitant K⁺ influx detected by the fluorescent probe potassium-binding benzofuran isophtalate. A rapid K⁺ influx ensues upon addition of valinomycin for both LAOOH (Fig. 5, trace a) and LA (trace c). The K⁺ influx associated with LAOOH-COO⁻ transport was inhibited by 2.5 mM ATP added to the assay medium (Fig. 5, trace b). In protein-free liposomes, no such charge transfer occurs with either LAOOH or LA (Fig. 5, traces d and e). The background K⁺ fluxes in the absence of UCP2 were similar to K⁺ fluxes obtained when 2.5 mM ATP was present inside and outside of the proteoliposomes containing UCP2 (not shown). These data indicate that the influx of hydroperoxy linoleic acid anions, detected as K⁺-influx-compensating anion charge influx, is faster than the influx of the linoleic acid anions. The sensitivity of these fluxes to ATP and their absence in liposomes indicates participation of UCP2.

Kineti cs of UCP2-catalyzed LAOOH-COO⁻ Unipor t as Compar ed with Linoleate Unipor t—By varying the total LAOOH concentration we evaluated the kinetics of hydroperoxylinoleate anion and linoleate uniport, as estimated from potassium-binding benzofuran isophtalate fluorescence in proteoliposomes containing UCP2 (Fig. 6). The obtained rates were corrected for fluxes in the absence of UCP2 and the net H⁺ fluxes were normalized to protein content. The apparent $K_m$ was 90 μM for LAOOH-COO⁻ and 350 μM for linoleate. The $V_{max}$ values were again similar, 18.6 and 17.0 μmol·min⁻¹·mg⁻¹ of protein⁻¹, respectively. In a series of three experiments, the calculated mean $K_m$ for the LAOOH-induced H⁺ fluxes was 90 ± 19 μM (S.D.) and the $V_{max}$ was ~20 μmol·min⁻¹·mg⁻¹ of protein⁻¹. The $K_m$ for linoleic acid was 364 ± 35 μM. These values are in good agreement with those obtained by measuring H⁺ fluxes.

**DISCUSSION**

We demonstrated that hydroperoxy derivatives of linoleic acid, like linoleic acid itself, are able to transport protons across the lipid bilayer by a flip-flop mechanism. LAOOH also supports uncoupling via UCP2, presumably by UCP2-mediated transport of its anionic form LAOOH-COO⁻ (38–46). In these respects, LAOOH behaves like other FAs, including its parent compound, linoleic acid. The finding that LAOOH is a substrate for UCP2 has several consequences. First, it suggests a feedback control mechanism by the liperoxidation products of increased ROS production, which stimulates UCP2-mediated uncoupling, which in turn might lead to the suppression of ROS production (7–11) (Fig. 7). Such stimulation may persist longer than the FA hydroperoxides because of their reduction by glutathione peroxidases to hydroxy-FAs (53), which likely also activate UCP2 to be its transport substrates. A second consequence of our results is that the proposed activation of UCP2 by
superoxide (33, 34) may in fact be because of a superoxide-induced increase in substrate for UCP2-mediated uncoupling. Echtay et al. (33, 34) originally proposed the direct interaction of superoxide with UCP2, but, as this was unlikely, they reformulated the sequence as a direct interaction of downstream products of lipid peroxidation, such as HNE (36). Note that the reported activation proceeded in the presence of FAs and that the reported activation proceeded in the presence of FAs or FAOOH or FAOH. Moreover, polyunsaturated (PU) FAs are themselves efficient substrates of UCP2 (32), so under conditions of elevated PUFAs, UCP2-mediated suppression of ROS production takes place as well. Note that like PUFAs (32), LAAOOH possesses a higher apparent affinity for the reconstituted UCP2 than major natural saturated or mono- or diunsaturated FAs.

The role of UCP2 in feedback down-regulation of ROS production by mitochondria may be important physiologically. UCP2 has been implicated in reducing the size of atherosclerotic plaques (29) and in influencing the lipoperoxidation state of low density lipoproteins when co-incubated with endothelial cells in which UCP2-induced uncoupling has taken place (28). UCP2 has also been implicated in improving brain recovery after stroke and preventing neurodegeneration (30) and playing a role in NO production by lipopolysaccharide-stimulated macrophages (27). These results support the importance of processes that regulate mitochondrial ROS production. Lipid peroxidation is a component of physiological and pathophysiological processes such as proliferation, inflammation, and aging (18). Enzymatic lipoperoxidation, in which PUFAs liberated by PLAA are oxidized by lipoxygenases, is an important component of cell signaling (15). An excess of PUFAs leads to the release of free iron ions from lipoxygenase and initiates further lipid peroxidation chain reactions that may induce apoptosis (18, 58, 59).

Despite the importance of feedback down-regulation of mitochondrial ROS production, the mechanism remains poorly understood. We suggest that the UCP2-mediated cycling of fatty acid hydroperoxides described here provides a plausible mechanism that is consistent with what is known about the UCP subfamily. In conclusion, two mechanisms were suggested so far for a feedback regulation by which ROS themselves suppress mitochondrial ROS production, (i) the mechanism Brand and co-workers (36) of direct but unexplained activation of UCP2 by certain ROS-derived activators and (ii) the cycling of fatty acid hydroperoxides (and products of their detoxification like hydroxy-fatty acids) mediated by UCP2 (Fig. 7). The UCP2 provides an efflux of their anions, which after protonation spontaneously flip-flop back across the membrane. This mechanism is more plausible, because FAOOH are liberated under lipid peroxidation and are degraded to FAOH, which are UCP2 cycling substrates as well.

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2 Experiments on isolated mitochondria are traditionally conducted at normal atmospheric pressure with isolated mitochondria contaminated by iron (in reagents or released from proteins) i.e. under highly oxidative conditions. By contrast, in situ mitochondria sense O2 levels as low as 0.3 kilopascals (2 torr, 3 µM) (54), iron traffic is limited and highly controlled, and cytosolic antioxidation mechanisms are present. The artificially established in vitro conditions when superoxide is externally developed (33) are thus far from the realistic in vivo conditions.
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