Rapid and Selective Oxygen-regulated Protein Tyrosine Denitration and Nitrination in Mitochondria*

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Growing evidence connects a cumulative formation of 3-nitrotyrosyl adducts in proteins as a marker for oxidative damage with the pathogenesis of various diseases and pathological conditions associated with oxidative stress. A physiological signaling role for protein nitration has also been suggested. Controlled “denitration” would be essential for such a contribution of protein nitration to cellular regulatory processes. Thus, we further characterized such a potentially controlled, reversible tyrosine nitration that occurs in respiring mitochondria during oxygen deprivation followed by reoxygenation, which we recently discovered. Mitochondria constitute cellular centers of protein nitration and are leading candidates for a “nitrative” regulation. Mitochondria are capable of completely eliminating 3-nitrotyrosyl adducts during 20 min of hypoxia-anoxia and undergoing a selective partial reduction after only 5 min. This denitration is independent of protein degradation but depends on the oxygen tension. Reoxygenation re-establishes protein tyrosine nitration patterns that are almost identical to the pattern that occurs before hypoxia-anoxia, with nitration levels that depend on the duration of hypoxia-anoxia. The identified mitochondrial targets of this process are critical for energy and antioxidant homeostasis and, therefore, cell and tissue viability. This cycle of protein nitration and denitration shows analogies to protein phosphorylation, and we demonstrate that the cycle meets most of the criteria for a cellular signaling mechanism. Taken together, our data reveal that protein tyrosine nitration in mitochondria can be controlled, is target-selective and rapid, and is dynamic enough to serve as a nitrative regulatory signaling process that likely affects cellular energy, redox homeostasis, and pathological conditions when these features become disturbed.

The covalent addition of a nitro group to protein tyrosine in vivo, forming 3-nitro-1-tyrosine, is a selective post-translational process targeting a few tyrosine residues in a limited number of proteins (1). Even though biological nitration yields are relatively low (100–500 μmol of nitrotyrosine/mol of tyrosine under inflammatory conditions) (2), they are an established marker for the extent of the production of nitric oxide (NO)1-derived reactive species during both physiological and pathological conditions. Cumulative protein tyrosine nitration, manifesting through multiple mechanisms, may be actively involved in the onset and/or progression of various diseases with an inflammatory component and associated with increased levels of reactive oxygen species and NO (1, 3–7). Cumulative protein tyrosine nitration may also be involved in the pathogenesis of acute pathological conditions such as ischemia-reperfusion (8, 9). The pathogenesis of these diseases and acute conditions may include alterations in tyrosine phosphorylation-dependent signal transduction (10) and/or mitochondrial dysfunction (11, 12) caused by protein nitration.

Mitochondria constitute a primary locus for intracellular superoxide radical generation and are exposed to NO by intramitochondrial production via a splice variant of neuronal nitric-oxide synthase (13) and extramitochondrial production via cellular nitric-oxide synthases (14). The simultaneous presence of superoxide and NO can lead to the formation of peroxynitrite, which is considered a source of protein nitration in mitochondria that is further enhanced by carbon dioxide or transition metals in proteins (Fig. 1) (14). The resulting high probability of protein nitration, especially in the mitochondrial matrix (15), is reflected by the fact that a substantial number of the proteins identified as nitrated in vivo are localized in mitochondria (6, 7).

In addition to the potential of protein nitration to impede protein function, recent data raise the issue of whether protein nitration might also be a cellular signaling mechanism (1, 16). To be considered a cellular signaling mechanism, protein nitration must meet four basic criteria: controlled rates of formation, specificity, modification of target protein and cell function, and reversibility (16). The specificity of protein nitration and modification of protein and cell functions by protein nitration have been demonstrated (1, 7, 15, 16); however, very little evidence exists regarding the reversibility by a protein “denitration” process in vivo (1, 16, 17). We recently discovered that isolated rat liver mitochondria are capable of nitrotyrosine clearance during a 20-min episode of hypoxia-anoxia and that nitrotyrosine immunoreactivity reappears during reoxygenation in a l-arginine-dependent way at protein spots that matched the spots before hypoxia-anoxia (18). Together with the fact that mitochondria constitute centers of nitration and data showing that mitochondria generate substantial amounts of reactive oxygen species (9, 19–21), NO (22, 23), and per-
oxy-nitrite (23, 24) during hypoxia-reoxygenation (ischemia-reperfusion), this finding suggested that the discovery of nitrotyrosine clearance might be part of a mitochondrial "nitrative" signaling mechanism.

In the present study, we show that mitochondrial nitrotyrosine clearance starts to take place within a few minutes, is selective during the early phase, depends on oxygen deprivation, and is not performed through proteolysis by contamination with proteasomes or lysosomes. We further demonstrate that nitration during reoxygenation is also selective and depends on the duration of hypoxia-anoxia and, therefore, on the degree of nitrotyrosine clearance. We identify the modified proteins, having verified that all main nitratated proteins before hypoxia-anoxia and after reoxygenation are identical, thus markedly increasing the possibility that nitrotyrosine clearance is indeed a nitration-denitration process involved in a nitrative-signaling mechanism. The metabolic pathways potentially affected by the nitrative signaling are shown.

***EXPERIMENTAL PROCEDURES***

**Isolation and Treatment of Mitochondria—** Rat liver mitochondria were isolated from adult Sprague-Dawley rats using differential centrifugation, as described previously in detail (25, 26), and preserved on ice until use, in solution at a concentration of 30 mg protein/ml. The respiratory control number of the preparations used in this study was 5.0 or above. The respiratory control number was determined using 3 mg/ml mitochondrial protein, 5 mM glutamate, 2.5 mM malate, and 1 mM ADP in a reaction medium (250 mM sucrose, 20 mM t-glucose, 40 mM KCl, 5 mM KH2PO4, 3 mM MgCl2, 0.5 mM EDTA, 30 mM Tris/HCl, pH 7.4, and protease inhibitors aprotinin (5 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and Pefabloc SC (24 μg/ml)) at 25 °C in isotemp oxygen electrodes. For hypoxia-anoxia-reoxygenation experiments (Fig. 2), 1.5 mM NADPH and 1 mM L-arginine or D-arginine were added. Hypoxia-anoxia was induced by sealing the chambers with the electrodes. At selected times, aliquots were removed and immediately oxidized using the Typhoon variable mode imager, and the images were processed and analyzed using ImageQuant Software, Amersham Biosciences.

**FIG. 1. Schematic illustration of nitration pathways in mitochondria.** The diffusion-controlled reaction of NO and superoxide (O2-) leads to the formation of peroxynitrite, which can react with carbon dioxide (CO2) or transition metals (Me) to form nitrogen dioxide (NO2) radicals, and carbonate radicals (CO3-) or the higher oxidation state of the transition metals (Me++-3-O). The one electron abstraction from protein tyrosine to yield protein tyrosyl radical (protein) occurs fast with both CO2 and Me++-1-O. Then the tyrosyl radical undergoes termination with NO. The nitration-denitration cycle is closed by an as yet uncharacterized denitration mechanism.

**FIG. 2. Schematic illustration of the experimental procedure of hypoxia-anoxia and reoxygenation.** A1, A2, and A3 mark the time points when aliquots were taken. Malate (Mlu), Glu, and ADP were added at the same time. 100% oxygen equals 297 nmol of oxygen/ml of respiratory buffer (51).

 strips, loading 145 μg of protein. Immobilized pH gradient strips were actively rehydrated with the sample at 50 V for 14 h, and then isoelectric focusing was performed by a linear increase to 250 V over 30 min followed by a linear increase to 8000 V over 1 h. The current was maintained at 8000 V until a total of 15 kVh was reached. For the second dimension, the immobilized pH gradient strips were equilibrated for 12 min in 50 mM Tris-HCl (pH 8.8), 6 mM urea, 30% glycero1, 2% SDS, 1% dithiothreitol, and bromphenol blue and then for 15 min in 50 mM Tris-HCl (pH 8.8), 6 mM urea, 30% glycerol, 2% SDS, 2% dioacetoamide, and bromphenol blue. The strips were then embedded in 1% (w/v) agarose on top of 12% acrylamide gels containing 4% stacking gel. The second dimension SDS-PAGE was performed essentially according to Laemmli (27). After SDS-PAGE, the acrylamide gels were soaked for 20 min in a transfer buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3, and 20% methanol) and then partially electro-transferred onto a PVDF membrane using a semidry transfer apparatus according to the manufacturer. The gels were then stained with colloidal Coomassie Blue (GelCode blue stain).

**Western Blot Analysis—** PVDF membranes were blocked for 60 min using blocking buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.2% Tween 20, and 1.5% bovine serum albumin). Membranes were probed for 60 min at 27.5 °C with monoclonal anti-nitryrosine antibody (1:5000 for two-dimensional gels, clone 1A6, Upstate Biotechnology, Lake Placid, NY), polyclonal anti-20 S proteasome antibody 1:2000 for one-dimensional gels, Biomol, Plymouth Meeting, PA), polyclonal anti-iso- citrate-1,4,5-triphosphate receptor type 1 (IP3R1) antibody (1:1000 for one-dimensional gels), monoclonal anti-histone H1 antibody (1:10000 for one-dimensional gels), and monoclonal anti-GM130 antibody (1:1000 for one-dimensional gels) or monoclonal anti-lysosome-associated membrane protein 2 (LAMP2) antibody (1:5 for one-dimensional gels) in blocking buffer and washed four times for 10 min in a washing buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.2% Tween 20). Anti-IP3R1, anti-histone H1, anti-GM130, and anti-LAMP2 antibodies were kind gifts of Dr. Alan Wolfman. Membranes were then probed for 60 min with a goat anti-mouse antibody (horseradish peroxidase conjugate, 1:8000; Sigma) and washed four times for 15 min in the washing buffer, and immunopositive spots were visualized by using ECL-Plus (Amer sham Biosciences) according to the manufacturer’s instructions. In experiments in which samples needed to be compared, the membranes were placed on the same film and exposed simultaneously.

**Two-dimensional Fluorescence Difference Gel Electrophoresis (Etten DlGE)—** DlGE CyDyes (Amersham Biosciences) were warmed to room temperature for 5 min, vortexed, and centrifuged for 30 s at 12,000 rpm. Then, 3 μl of CyDyes were mixed for 30 min in the dark with 4.5 μl of anhydrous dimethylformamide to reach a concentration of 400 μg 50 μg of mitochondrial protein from samples taken before 20 min of hypoxia-anoxia and after 20 min of reoxygenation were separately labeled with DlGE CyDye Cy5 (blue) and Cy3 (red), respectively. The reaction was stopped by adding 1 l of 10 mM lysine, and the samples were vortexed and centrifuged for 30 s at 12,000 rpm. Both samples were mixed together, and proteins were separated by two-dimensional gel electrophoresis as described previously. Labeled proteins were visualized using the Typhoon variable mode imager, and the images were analyzed (ImageQuant Software, Amersham Biosciences).
Protein Identification—Nitrotyrosine immunoreactive protein spots were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometric analysis of in-gel tryptic digest of nitrotyrosine immunopositive spots as described previously in detail (6). All searches were performed with a mass tolerance of 0.005% error (50 ppm) and a minimum of four matching peptides. To verify antibody specificity for nitrotyrosine, proteins transferred onto PVDF membrane were tested for nitrotyrosine immunoreactivity after reduction with sodium hydrosulfite (6).

Quantification of Total Nitrotyrosine and Tyrosine—The snap-frozen samples were thawed and analyzed by stable isotope dilution high pressure liquid chromatography-tandem mass spectrometry, as described previously in detail, using 300 μg of protein per analysis (28).

RESULTS AND DISCUSSION

The influence of hypoxia-anoxia followed by reoxygenation (for a schematic view, see Fig. 2) on protein nitration was tested in isolated, intact rat liver mitochondria respiring at respiration state 3. The rat liver preparations showed no contamination with nuclei (using histone H1 as a marker) (29), Golgi (GM130 as marker) (30), lysosomes (LAMP2 as marker) (31), and 20 S proteasomes that are found in nuclear, cytosolic, and microsomal preparations isolated from rat liver (32) (data not shown). Freshly isolated mitochondria showed a background protein tyrosine nitration that was consistent for all preparations. L-arginine (1 mM) was used to support NO generation (33, 34) during hypoxia and reoxygenation. Compared with the protein nitration pattern of freshly isolated mitochondria (control) (Fig. 3a), the patterns after a hypoxic-anoxic phase of 5 min (approximately 2 min of anoxia) (Fig. 3b) or 20 min (approximately 17 min of anoxia) (Fig. 3c) both demonstrated extensively decreased intensities of nitrotyrosine immunoreactivity. Five minutes of hypoxia resulted in a selective alteration of the nitration pattern. The decrease in signal intensity for most of the immunoreactive pro-
tein spots ranged from approximately 25 to 75% (Fig. 3b, spots S1, S2, S4, and S5). The signals of other immunoreactive proteins almost completely disappeared (Fig. 3b, spot S3). After 20 min of hypoxia-anoxia, the mitochondria showed a complete loss of nitrotyrosine immunoreactivity. It was shown recently (33) that, in the absence of protease inhibitors, the removal of nitrated proteins in isolated mitochondria is caused by increased turnover, with protein half-lives decreasing from 142–14 h to 3.8–0.5 h. within 0.5 h. In the present study, however, a substantial decrease in nitrotyrosine immunoreactivity occurred within a few minutes and in the presence of protease inhibitors. Moreover, we verified the absence of 20 S and, therefore, 26 S proteasomes as well as lysosomes. The 20 S proteasome appears to be the predominant cellular pathway for the degradation of soluble and membrane-bound, mildly oxidized proteins (proteins with <9 modified amino acids), alone and in cooperation with the lysosomal system (35, 36). Therefore, if present, the 20 S proteasome would be a prime candidate for the observed loss of nitrotyrosine immunoreactivity because it selectively recognizes and degrades present oxidized proteins, even during ongoing oxidative stress as in hypoxia and reoxygenation (35, 36). Mitochondria contain an autonomous proteolytic system, which includes ATP-dependent Lon and Lon-like serine proteases in the matrix as well as two ATP-dependent AAA metalloproteases that are an integral part of the inner membrane (37). Among these proteases, degradation of oxidatively modified proteins has been shown only for the Lon protease (38), which is inhibited by serine protease inhibitors such as phenylmethylsulfonyl fluoride, even in the presence of the activator ATP (39). Therefore, Lon and Lon-like proteases should be inhibited by the Pefabloc SC that was added to all mitochondrial preparations. Thus, the clearance of the nitrotyrosine immunoreactivity is more likely performed by a protein denitration process then by protein degradation.

The steady state abundance of the proteins in the isolated mitochondria generally depends on protein degradation and mitochondrial protein synthesis; however, except for the AAA metalloproteases, all known mitochondrial proteases were inhibited, and the mitochondrial protein synthesis is limited to proteins encoded in the mitochondrial DNA, which are all subunits of enzyme complexes of the oxidative phosphorylation system (40). To test for any alteration in the abundance of the mitochondrial proteins and especially for remaining proteolytic degradation by mitochondrial proteases, we performed two-dimensional fluorescence difference gel electrophoresis (Fig. 4). We compared samples before hypoxia-anoxia (Fig. 4a, blue fluorescent protein spots) and after 20 min of reoxygenation (Fig. 4a, red fluorescent protein spots). No differences in protein expression patterns and levels were observed (Fig. 4a, overlay of the two samples shown). This further raises the probability that a protein denitration process is responsible for the loss of nitrotyrosine immunoreactivity, but because of the limitations of the method, a specific degradation of the nitrated proteins by an unidentified protease at subdetection limits (between 1 and <10%) cannot be fully ruled out.

In contrast to the mitochondrial denitration observed in our experiments, total protein nitration in whole organs increases during ischemic hypoxia (9), which is consistent with amplified cellular levels of superoxide in the presence of NO and CO2 (14, 19, 20, 22, 41). Thus, mitochondrial nitrotyrosine clearance may be part of an organelle-specific defense mechanism against oxidative and nitrosative stress under conditions of decreasing oxygen tension, because one of the main sources of reactive oxygen species are complexes I–III of the mitochondrial respiratory chain (42). If a protein denitration mechanism is responsible for this clearance of nitrotyrosine, it likely involves either reduction of nitrotyrosine to aminotyrosine or full removal of the nitro group from the tyrosine residue. Nitrotyrosine may be chemically reduced to aminotyrosine (43), but removal of the nitro group would require an enzymatic process (17, 44).

Reoxygenation of oxygen-deprived mitochondria resulted in altered protein nitration (Fig. 3, d–f). To examine the contribution of NO to the nitration process, we exposed mitochondria to hypoxia-anoxia and reoxygenation in the presence of either L-arginine or D-arginine. D-arginine is not a substrate for mitochondrial nitric-oxide synthase (45), and neither L-arginine nor D-arginine affects the transmembrane potential or the oxygen consumption at respiration state 3, at the concentrations used (data not shown) (46). In the presence of L-arginine, nitrotyrosine immunoreactivity changed relative to the duration of hypoxia-anoxia (Fig. 3, d and e), whereas in the presence of D-arginine, reoxygenation resulted in virtually no increase in nitrotyrosine immunoreactivity (Fig. 3f), verifying that NO synthesis is essential for protein nitration during reoxygenation. Reoxygenation in the presence of L-arginine after the incomplete denitration during 5 min of hypoxia-anoxia resulted in selective nitration, with almost unchanged nitrotyrosine immunoreactivity for some proteins (compare Fig. 3, d and b, spots S2 and S4) and increased nitrotyrosine immunoreactivity for other proteins as well (compare Fig. 3, d and b, spots S1, S3, and S5) in a few immunoreactive spots seen only after reoxygenation (Fig. 3, d and e, spot S6, arginase 1). Reoxygenation after the complete denitration during 20 min of hypoxia-anoxia resulted in nitrotyrosine immunoreactivities that were generally notably higher by approximately 150% (Fig. 3e) than the immunoreactivities in mitochondria before hypoxia-anoxia (Fig. 4, a and b).
The presence of L-arginine, which suggests that the denitration process occurred in the presence of oxygenated mitochondria increased over a period of 20 min in the presence of L-arginine (L-Arg HA) or D-arginine (D-Arg OX), and after 20 min of reoxygenation following 20 min of hypoxia-anoxia (L-Arg HA-RO). Significant alterations are shown before hypoxia-anoxia that matched the nitrotyrosine immunoreactive spots on the autoradiograph in Fig. 3a (proteins corresponding to spots selected as S1–S5 are indicated). Proteins were identified as 5, manganese superoxide dismutase (S1, sequence coverage 32%); 6, electron transfer flavoprotein β-chain (S5, sequence coverage 32%); 7, enoyl-CoA hydratase (sequence coverage 20%); 8, electron transfer flavoprotein α-chain (S3, sequence coverage 20%); 9, malate dehydrogenase (S5, sequence coverage 35%); 10, and 7, acetyl-CoA acyltransferase (S4, sequence coverage 32%); 11, mitochondrial aldehyde dehydrogenase (S4, sequence coverage 13%); and 9, arginase 1, which is nitratated only after reoxygenation (corresponds to nitrotyrosine immunoreactive S6 in Fig. 3, d and e, sequence coverage 39%).

As an approach to establish potential functions for the rapid and selective denitration-nitration process and to verify the results from the Western blot analysis with the anti-nitrotyrosine antibody, we identified the proteins that are nitratated under the different conditions shown in Fig. 3. All Coomassie-stained protein spots of the gels (Fig. 6, mitochondria are shown before hypoxia-anoxia) that matched the nitrotyrosine immunoreactive spots in the autoradiographs were analyzed. Corresponding nitrotyrosine immunoreactive spots from the different conditions were identified as the same proteins. It is not clear, however, if certain protein molecules undergo a full denitration-nitration cycle or if different molecules of the same protein are denitrated during hypoxia-anoxia and nitratated during reoxygenation.

Among these reversibly nitratated proteins are a major antioxidant enzyme (manganese superoxide dismutase), two proteins involved in fatty acid β-oxidation (enoyl-CoA hydratase and acetyl-CoA acyltransferase), a key electron acceptor for multiple pathways (electron transport flavoprotein), and a protein of the citric acid cycle (malate dehydrogenase). Thus, in addition to the mitochondrial antioxidant defense that is potentially altered by nitrative inhibition of manganese superoxide dismutase (48), major mechanisms of energy homeostasis, including the electron transfer between various flavoprotein dehydrogenases (amino acid and choline catabolism) and the respiratory chain (see Ref. 49 and references therein), are affected (Fig. 7). The denitration of these enzymes during the hypoxic-anoxic phase likely helps to sustain mitochondrial ATP production and an increased antioxidant capacity, which is important for the prevention of metabolic failure and cell death during oxygen deprivation. In this context, it is important to note that damage caused by oxygen deprivation in tissues depends on the duration of hypoxia or anoxia, with 5 min of hypoxia-anoxia conferring protective ischemic preconditioning (24) and extended hypoxia-anoxia causing severe tissue injury during reperfusion (50). The periods of hypoxia-anoxia and reoxygenation applied in this study cover the conditions for both ischemic preconditioning and extended hypoxia-anoxia. Our data suggest that the partial denitration and/or partial de novo nitration observed after 5 min of hypoxia-anoxia and the
Mitochondrial metabolic pathways affected by nitration. The identified nitrated proteins are highlighted (italic bold). Their metabolic functions within the different pathways suggest that fatty acid β-oxidation and the citric acid cycle, as well as amino acid and choline catabolism, are affected. Alterations within these metabolic pathways may also alter the electron transfer to the respiratory chain. ADH, acyl-CoA dehydrogenase; ADH, alcohol dehydrogenase; ADP, adenosine diphosphate; ADP/ATP translocase; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocopherol; ATR, alpha-tocophero
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