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Analysis of Monohydroxyeicosatetraenoic Acids and F_2-isoprostanes as Markers of Lipid Peroxidation in Rat Brain Mitochondria

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We have introduced two specific techniques for the quantitative measurement of monohydroxyeicosatetraenoic acids (HETEs) and F_2-isoprostanes by gas chromatography–mass spectrometry/negative ion chemical ionization (GC–MS/NICI) to study lipid peroxidation in isolated rat brain mitochondria by iron/ascorbate. The analysis of HETEs involved hydrogenation, solid phase extraction on a C_{18}-cartridge, formation of pentafluorobenzyl bromide and trimethylsilyl ether derivatives. In the case of F_2-isoprostanes, the analytical procedure was similar to that of HETEs except that the hydrogenation step was omitted. We found that HETE content (sum of 5-, 8–12-, and 15-isomers) in freshly prepared rat brain mitochondria was 220 ± 40 pmol/mg protein. The corresponding content for the F_2-isoprostane, 8-iso-$PGF_{2\alpha}$, was 0.21 ± 0.10 pmol/mg protein. HETEs and 8-iso-$PGF_{2\alpha}$ were predominantly present in the esterified form. The content of both HETEs and 8-iso-$PGF_{2\alpha}$ were increased in presence of iron/ascorbate as oxidation system. After 30 min incubation with Fe^{2+}+ ascorbate, the content of HETE isomers was increased about 6-fold compared with baseline levels whereas that for 8-iso-$PGF_{2\alpha}$ was elevated 100-fold. Formation of HETEs and F_2-isoprostanes corresponded to the consumption of arachidonic acid (AA) and α-tocopherol, respectively. There were almost no changes in the content of free (non-esterified) HETEs and 8-iso-$PGF_{2\alpha}$ during the course of iron/ascorbate induced oxidation of the brain mitochondria. Our data provide the first direct evidence for the presence of HETEs and F_2-isoprostanes in freshly isolated rat brain mitochondria and that esterified HETEs and 8-iso-$PGF_{2\alpha}$ are predominantly generated during iron/ascorbate induced lipid peroxidation. Sensitive quantification of these products of non-enzymatic lipid peroxidation as indicators of oxidant injury opens new areas of investigation regarding the role of free radicals in the pathogenesis of human diseases. In addition, HETEs and F_2-isoprostanes may be important mediators for mitochondrial functions.

Keywords: HETEs; F_2-isoprostanes; Lipid peroxidation; Gas chromatography–mass spectrometry; Rat brain mitochondria

Abbreviations: HETEs, monohydroxyeicosatetraenoic acids; GC–MS, gas chromatography–mass spectrometry; NICI, negative ion chemical ionization; SIM, selected ion monitoring; TBARS, thiobarbituric acid reactive substances; BSA, bovine serum albumin; ROS, reactive oxygen species; PUFA, polyunsaturated fatty acid; AA, arachidonic acid; DHA, docosahexaenoic acid; LA, linoleic acid; PFB, pentafluorobenzylbromide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; 12-HHT, 12-hydroxyheptadecatrienoic acid; BHT, butylated hydroxytoluene; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; MDA, malonic dialdehyde

INTRODUCTION

Oxidative mechanisms, especially lipid peroxidation, play an important role in the pathogenesis of many human diseases, such as Alzheimer's disease, Parkinson's disease, atherosclerosis, inflammation or ischemia. The development of specific, reliable and sensitive quantitative methods for the characterization of oxidative stress in humans is essential for establishing the involvement of free radicals in the development of these pathologies.[1]

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Despite a great deal of effort, accurate techniques to assess oxidant injury are limited.

Peroxidation of polyunsaturated fatty acids (PUFAs) results in the formation of conjugated dienes, hydroperoxides, hydroxy fatty acids, PGF-like compounds as well as mono- and bifunctional aldehydes and volatile hydrocarbons.[2] Fatty acid hydroperoxides are the primary peroxidation products. These are either reduced to the corresponding hydroxy derivatives mainly mediated by glutathione peroxidases[3] or converted to other secondary lipid peroxidation products.[2] Fatty acid hydroperoxides can be measured directly and indirectly by a variety of techniques.[4–6]

Arachidonic acid (AA) as one of the most abundant PUFAs in cell membranes is susceptible to free radical attack with abstraction of an allylic hydrogen atom from a reactive methylene group to form a dienyl radical. The resulting free radical has multiple resonance structures that increase its stability. Reaction of these resonance forms with oxygen fixes the double bonds in a conjugated arrangement to produce peroxyl radicals.[7] Peroxidation of AA occurs both enzymatically and non-enzymatically. In the case of enzymatic oxygenation in presence of lipoxygenases and cytochrome P-450, the removal of hydrogen and the introduction of molecular oxygen are stereoselective leading to the formation of regio- and stereospecific products. Formation of 5-, 8-, 12-, or 15-HETEs is favored during reactions catalyzed by lipoxygenases.[8] During free radical-induced oxidation of AA all oxidizable positions may be attacked and a complex product mixture of different positional and optical isomeric racemic products is formed.[9]

F_2-isoprostanes are a series of chemically stable end products of free-radical mediated peroxidation of AA. They are isomeric with enzymatically formed eicosanoids, such as prostaglandin F_2. F_2-isoprostanes are widely used as biomarkers of lipid peroxidation in both humans and experimental animal models. They consist of four main classes of regio-isomers, each consisting of eight possible diastereomers. 8-iso-$PGF_{2\alpha}$ (i-$PGF_{2\alpha}$-III or formerly termed 8-epi-$PGF_{2\alpha}$) has attracted most attention among the F_2-isoprostanes formed *in vivo*. F_2-isoprostanes are formed *in situ* in phospholipids and are subsequently released by phospholipases in contrast to prostaglandins which are generated from free AA.[10–12]

The measurement of HETEs and F_2-isoprostanes by GC–MS has opened a new avenue to quantify lipid peroxidation more specifically with higher sensitivity. In addition to being markers of oxidative stress they possess biological activities and in some cases have been adopted as regulated second messengers.[11,13,14]

The aim of the present work was to introduce and to compare two sensitive and specific GC–MS methods for the quantification of free and esterified HETEs and F_2-isoprostanes as measures of oxidative stress in isolated rat brain mitochondria using iron/ascorbate as oxidizing agent. The utilization of iron/ascorbate for the induction of lipid peroxidation has sufficiently been characterized for isolated rat liver and heart mitochondria in the past.[15,16] This experimental model displays a lag-phase in the formation of MDA characterized by progressive deenergization of mitochondria and exhaustion of antioxidants, followed by an exponential and massive increase in lipid peroxidation assayed by formation of TBARS.

In the present work, we have analyzed the content of HETEs and F_2-isoprostanes in freshly isolated rat brain mitochondria and during iron/ascorbate induced lipid peroxidation. HETEs and F_2--isoprostanes are originally derived from the mostly occurring PUFA (20:4) in brain mitochondria. The data should be related to: (i) common, more unspecific secondary markers of lipid peroxidation (TBARS); (ii) the loss of PUFAs, preferentially AA; and (iii) the exhaustion of α-tocopherol as an important lipid-soluble mitochondrial antioxidant. The sensitive gas chromatography–mass spectrometry-based methods for quantitation of HETE and isoprostane isomers would open new opportunities to study the mechanism of lipid peroxidation as well as possible biological activities of these oxidized products in mitochondria. If they are formed in significant amounts in membrane-bound phospholipids, they may contribute to modify metabolic events, such as ATP production, ion transport, expression of cytokines and apoptosis.[11,17–21]

MATERIALS AND METHODS

Preparation of Functionally Intact Rat Brain Mitochondria

Mitochondria were prepared from the brains of Wistar rats about 2 months old in ice-cold medium containing 250 mM mannitol, 20 mM TRIS, 1 mM EGTA, 1 mM EDTA and 0.3% (w/v) bovine serum albumin (BSA) at pH 7.4 (isolation medium) using a standard procedure.[22] After the initial isolation, Percoll was used for purification of mitochondria containing residual endoplasmic reticulum, Golgi apparatus and plasma membranes. The mitochondria were well coupled, as indicated by a respiratory control index greater than four with glutamate plus malate as hydrogen supplying substrates.

Iron/ascorbate Induced Lipid Peroxidation and Determination of TBARS

The mitochondrial suspension (1 mg protein/ml) was incubated in a medium containing 0.1 M KCl and

0.01 M TRIS (pH 7.4) in the presence of 50 μM iron II-sulfate and 500 μM ascorbate by continuous shaking in an open air atmosphere. Aliquots were removed at time intervals and mixed with butylated hydroxytoluene (BHT) as chain-breaking antioxidant at a final concentration of 100 μM. The samples were immediately frozen in liquid nitrogen, stored at −80°C and subsequently assayed for the content of HETEs, F_2-isoprostanes, α-tocopherol and fatty acid composition. The TBARS determination was performed immediately according to Buege and Aust[23] and to Jentzsch et al.[24] The TBA-MDA-complex was separated by C18-reversed phase-HPLC (eluent: 50 mM KH_2PO_4, pH 7.0 and 35% CH_3OH, flow rate: 1.0 ml/min) and detected as compared to the authentic standard (tetramethoxypropane) at 532 nm.[25]

Quantification of HETEs

Free (Non-esterified) HETEs

For quantification of free HETEs the method of Thomas et al.[26,27] was used with the modification described recently by our group for plasma samples.[28] In brief, 10 ng of 12-hydroxy-heptadecatrienoic acid (12-HHT) in 20 μl ethanol were added as the internal standard to 100 μl of mitochondrial samples (1 mg protein/ml). The absence of this compound in rat brain mitochondria was examined and confirmed under all experimental conditions applied. Hydrogenation was carried out using PtO_2/H_2 in methanol/ethyl acetate (50/50, v/v) at 60°C for 15 min. Subsequently, the solvents were removed under argon and 4 ml of 0.1 M HCl were added and the samples were applied onto C_{18}-cartridge pre-washed with 5 ml of methanol and water. The cartridge was then washed with 10 ml of 0.1 M HCl and 10 ml of acetonitrile/water (15/85, v/v). HETEs were eluted by washing the column with 5 ml of hexane/ethyl acetate/2-propanol (30/65/5, v/v/v).

Total (Sum of Free and Esterified) HETEs

10 ng of 12-HHT in 20 μl ethanol were added as the internal standard to 100 μl of mitochondrial preparations (1 mg protein/ml) and hydrogenation was carried out as described above. Esterified lipids were cleaved by incubating the samples with 50 μl of 5 M KOH at 40°C for 30 min and acidified using 200 μl of 1 M HCl and 4 ml of 0.1 M HCl. Finally, HETEs were isolated by chromatography on a C_{18}-cartridge as described above.

Pentafluorobenzyl Ester/trimethylsilyl Ether Derivatization

Extracts following C_{18} chromatography step were dried under a stream of argon at 40°C. 10 μl of PFB, 5 μl of N,N-diisopropylethylamine and 50 μl of dichloromethane were added to the residues and the samples were kept at 60°C for 15 min. The samples were dried under a stream of argon at 40°C. 50 μl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), 10 μl of N,N-diisopropylethylamine and 20 μl of dichloromethane were added. The samples were kept at 40°C for 60 min and the solvents were removed under a stream of argon. Finally the sample was dissolved in 100 μl dichloromethane (containing 0.1% BSTFA).

Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry/negative ion chemical ionization (GC–MS/NICI) was carried out on a Varian GC linked to a SSQ 710 Finnigan MAT (Bremen, Germany) using the NICI with methane as reagent gas. Samples (0.5 μl) were injected into a temperature programmed Gerstel injector (KAS 3, Mülheim, Germany) onto a DB 5-MS column (30 m × 0.25 mm ID; 0.25 μm film thickness; J&W Scientific, Folsom, CA). Separation was carried out using the following temperature program: Initial temperature 180°C for 2 min; with rate 10°C/min to 260°C; with rate 5°C/min to a final temperature of 285°C, maintained for 15 min; final time: 30 min. Quantitative analysis was performed using selected ion monitoring (SIM) of the carboxylate anion $[M-181]^-$ at m/z 399 and 357 for HETEs and 12-HHT, respectively. The response factors for the different HETEs were 1.00 ± 0.08 for all isomers (five separate determinations).

Quantification of F_2-isoprostanes

Free (Non-esterified)

Determination of F_2-isoprostanes was basically carried out as described by Nourooz-Zadeh et al.[29] Briefly, 12.5 ng of $PGF_{2\alpha}$-d_4 in 25 μl ethanol as internal standard was added to 250 μl of mitochondrial suspension (1 mg/ml). The pH was adjusted to 2 using about 500 μl of 0.1 M HCl, subsequently five volumes of ethyl acetate were added and the samples were vortex-mixed for 30 s. The samples were centrifuged at 2000 g for 5 min and the organic (upper) layer was transferred into a new glass tube. The total lipid extract then was applied onto a NH_2-cartridge pre-washed with hexane (5 ml). The columns were subsequently washed with 10 ml of hexane/ethyl acetate (30/70, v/v), acetonitrile/water (90/10, v/v) and acetonitrile. F_2-isoprostanes were eluted washing the column with 5 ml of ethyl acetate/methanol/acetic acid (10/85/5, v/v/v).

Total (Sum of Free and Esterified)

250 µl of mitochondrial preparations (1 mg protein / ml) were incubated with 250 µl of 4 M KOH at 40°C for 30 min to cleave esterified lipids. The pH was adjusted to pH 2 using about 250 µl of 4 M HCl and $PGF_{2\alpha}$-d_4 as internal standard was added. Total lipid extraction and NH_2-chromatography were carried out as described above.

Pentafluorobenzyl Ester/trimethylsilyl Ether Derivatization and GC–MS/NICI Analysis

Final lipid extracts following NH_2-chromatography step were dried under a stream of argon at 45°C. 40 µl of PFB (10% in acetonitrile) and 20 µl of N,N-diisopropylethylamine (10% in acetonitrile) were added to the residues and the samples were kept at 45°C for 30 min.

50 µl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 5 µl of N,N-diisopropylethylamine were added to the dried sample. The samples were kept at 45°C for 45 min, the solvents were removed and the samples were dissolved in 40 µl isooctane (containing 0.1% BSTFA). F_2-isoprostanes were separated using the following temperature program: initial temperature of 175°C for 2 min; with rate 30°C / min to final temperature of 270°C maintained for 27 min; final time: 32.2 min. Quantitative analysis was performed using SIM of the carboxylate anion $[M-181]^-$ at m/z 569 and 573 for F_2-isoprostanes and $PGF_{2\alpha}$-d_4, respectively. The response factors for the isoprostane isomers were 1.00, 0.65 and 0.55 for 9α,11α-, 9α,11β- and 8-iso-$PGF_{2\alpha}$, respectively (means of three separate determinations).

Fatty Acid Analysis

Fatty acid profile of the brain mitochondrial samples was determined by the analysis of total fatty acids as their methyl esters. 200 µl (1 mg protein / ml) of mitochondrial suspension were mixed with 250 µl (100 µg in ethyl acetate) of heptadecanoic acid as an internal standard. 800 µl water and 750 µl of ethyl acetate were then added to the homogenate, vortexed and centrifuged at 2500 g for 5 min. The upper (organic) layer was then transferred to a clean glass vial. A further 500 µl of ethyl acetate were added to the remaining aqueous phase, vortexed and centrifuged. The organic layers were pooled and dried down under a stream of nitrogen. Fatty acid methyl esters were prepared by adding 500 µl of boron trifluoride-methanol (14%) solution to the dried lipid, capped and incubated at 60°C for 30 min. The samples were allowed to cool before 500 µl water and 1 ml hexane were added. The mixtures were vortexed and centrifuged. The hexane layer was collected and dried. The residue was re-dissolved in 100 µl of hexane, of which 1 µl was injected onto an Omegawax™ 320 column (0.32 µm × 30 m, film thickness 0.25 µm) using a temperature gradient of 120–240°C at 6°C / min. The signal was detected by a flame ionization detector.[12]

α-Tocopherol and Protein

α-Tocopherol was measured by HPLC after extraction in hexane, according to Lang et al.,[30] modified by Noack et al.[31] Protein content was measured according to the method of Lowry et al.[32] using BSA as a standard.

RESULTS

Initiation of Lipid Peroxidation in Brain Mitochondria and Fatty Acid Content

Oxidative stress was induced in freshly isolated, functionally intact rat brain mitochondria by exposure to iron / ascorbate. The extent of lipid peroxidation was routinely assessed by the measurement of TBARS and depletion of PUFAs including LA, AA, and DHA. As shown in Fig. 1, exposure of the mitochondria to iron / ascorbate led to a time-dependent accumulation of TBARS with a maximum at about 30 min. This coincided with the consumption of AA, DHA and vitamin E (Fig. 1). Arachidonic and docosahexaenoic acids were the most abundant PUFA in rat brain mitochondria. They accounted for about one third of total fatty acids (not shown). Linoleic acid, the major fatty acid of mitochondrial cardiolipin is less abundant and obviously did not change during the time course of lipid peroxidation.

Quantification of HETEs

HETEs were measured as PFB-ester / TMS-ether derivatives with NICI by SIM. Figure 2 shows a typical GC–MS chromatogram of HETEs in mitochondrial samples, in which 2-, 3- and 15-HETE are identified unambiguously, whereas the HETEs with hydroxy groups in the positions 5, 8 + 9 and 11 + 12 were not completely resolved. Quantification of the partially overlapped HETEs was carried out manually for each sample and 8–12-HETEs were taken together as one fraction.[28] Identification of HETEs was based on the comparison of relative retention times of the components in the samples to those of authentic standards. Chemical structure was verified by positive ion chemical ionization, answering the question of the location of the hydroxy group via specific fragment ions after α-cleavage. Positive ion chemical ionization spectrum of 5-HETE is shown in Fig. 3.

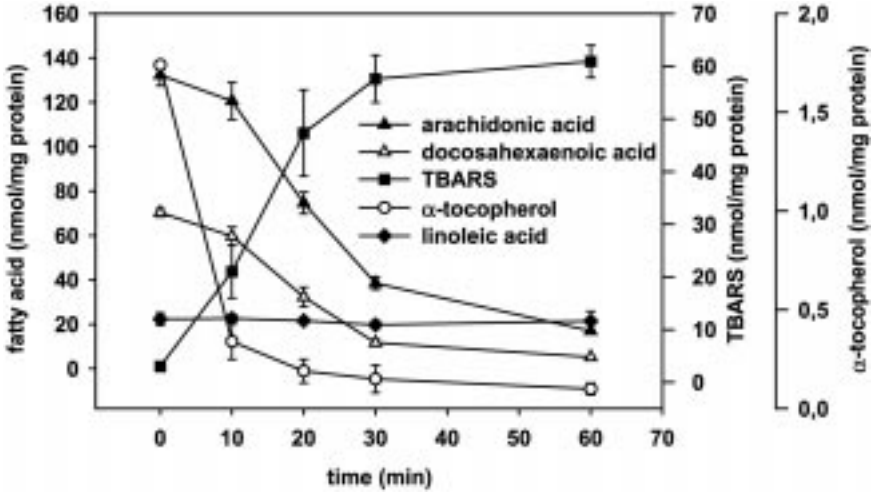

FIGURE 1 Decline of PUFAs and α-tocopherol and formation of TBARS during iron/ascorbate induced peroxidation. α-Tocopherol content in mitochondria before the initiation of oxidative stress was 1.7 nmol/mg mitochondrial protein (values are means ± SE of three experiments).

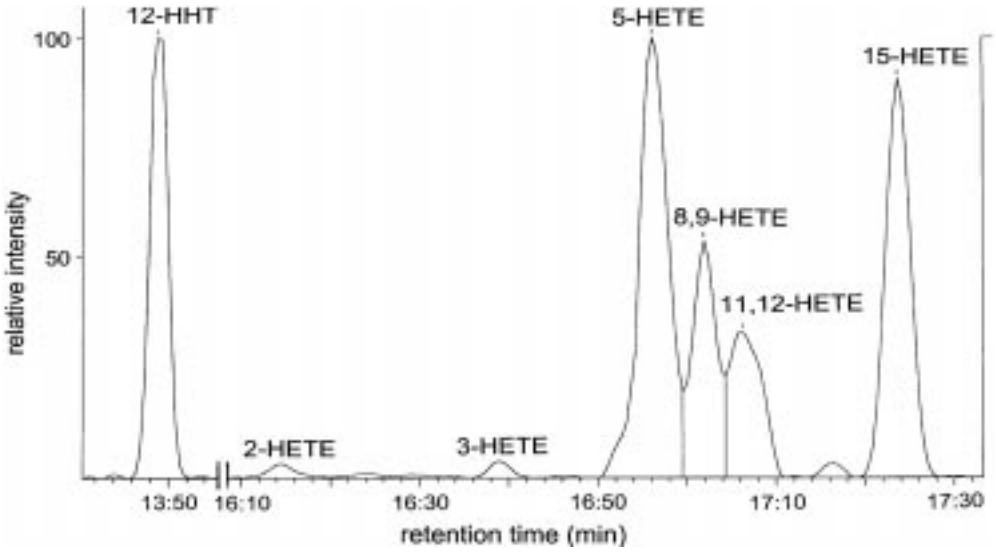

FIGURE 2 Representative GC–MS/NICI chromatogram showing the typical pattern of HETEs in rat brain mitochondria after 30 min peroxidation using iron/ascorbate. The analysis was performed using SIM at m/z 399 (HETEs) and 357 (12-HHT).

The time course for the formation of individual (sum of free and esterified) HETEs (5-, 8–12- and 15-HETEs) during initiation of lipid peroxidation by iron/ascorbate in the rat brain mitochondria is shown in Fig. 4. Total formation of HETEs was increased with time and reached a maximum at about 30 min. 5-, 8–12- and 15-HETEs were the major components formed during oxidation of the brain mitochondria in the presence of iron/ascorbate, whereas 2- and 3-HETEs were found to be minor components (Fig. 2). Basal content of total 5-, 8–12- and 15-HETE isomers in the mitochondrial preparations was 220 ± 40 pmol/mg protein (Table I). This accounts for about 0.3% of originally available AA. After 30 min incubation with iron/ascorbate, the content of total 5-, 8–12- and 15-HETE isomers in the mitochondria was increased by 5.6-fold (1.2 nmol/mg protein). Individual HETEs contributed to this enhancement with different shares.

There were only minor changes in the content of free (non-esterified) HETEs including 5-, 8–12- and 15-isomers during the course of incubation. The content of free and esterified 5-, 8–12- and 15-HETE isomers following 30 min oxidative stress is summarized in Table II.

Quantification of F_2-isoprostanes

Oxidation of rat brain mitochondria in the presence of iron/ascorbate resulted in the accumulation of a family of PGF_2-like compounds with 8-iso-$PGF_{2\alpha}$, 9α,11β-$PGF_{2\alpha}$ and 9α,11α-$PGF_{2\alpha}$ as reliably identified isomers. Figure 5 shows a typical GC–MS/NICI chromatogram of total (sum of free and esterified) PGF_2-like compounds

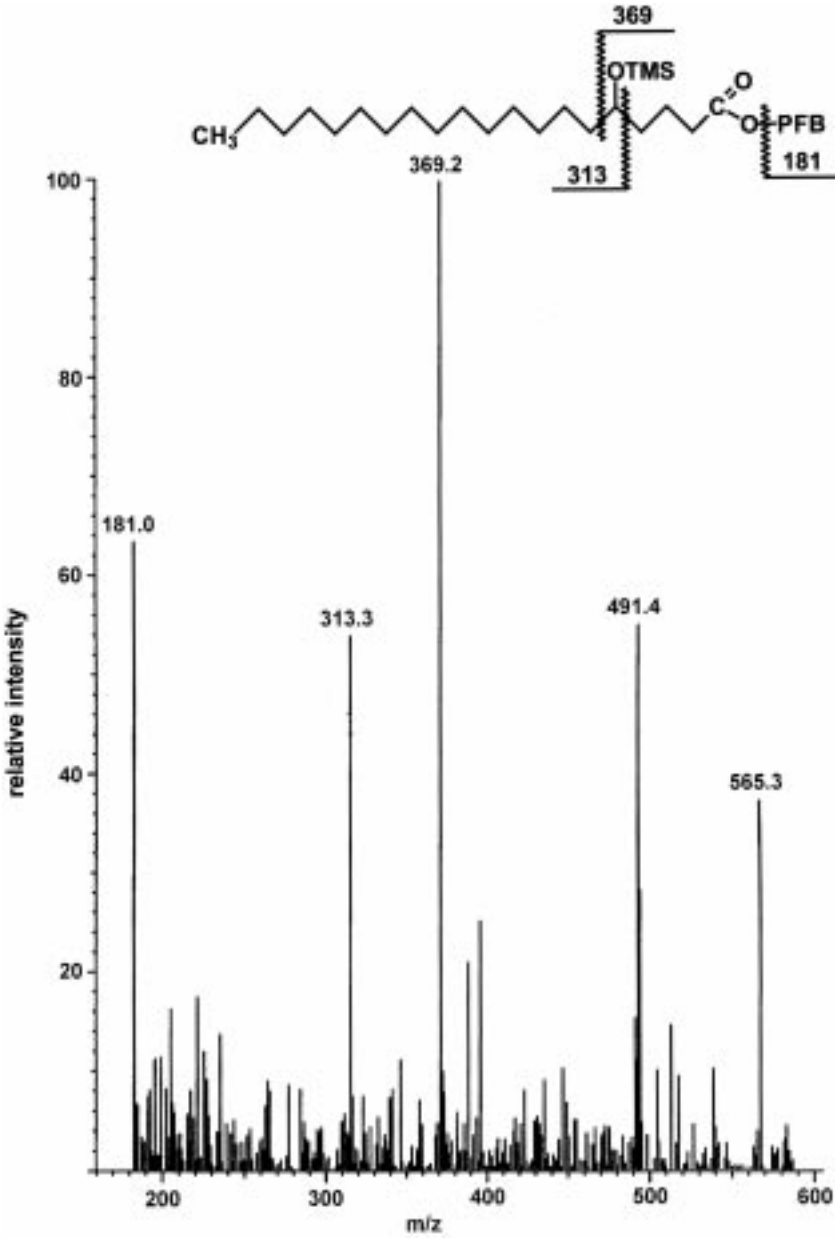

FIGURE 3 GC–MS/PICI mass spectrum of 5-HETE (as 5-hydroxyeicosanoic acid-pentafluorobenzyl ester-trimethylsilyl ether). Demonstration of α-cleavage fragments (m/z: 313 and 369, respectively).

in oxidized mitochondrial preparation. The upper chromatograms (a and b) represent the PGF_2-like compounds (mitochondrial sample and authentic standards) monitored at m/z 569 while that monitored at m/z 573 (c, lower chromatogram) represents the tetradeuterated $PGF_{2\alpha}$ as the internal standard. There were nearly no or minor changes in basal levels of 8-iso-$PGF_{2\alpha}$ and the other isoprostanes for up to 10 min following oxidation of the mitochondria with iron/ascorbate (Fig. 6). Thereafter, the formation of 8-iso-$PGF_{2\alpha}$ was increased rapidly, but time-delayed compared to HETEs, and reached a maximum level after about 45 min. The content of free 8-iso-$PGF_{2\alpha}$ was extremely small and remained nearly unchanged over the whole time course (Fig. 7). Basal levels of esterified 8-iso-$PGF_{2\alpha}$ were 0.21 pmol/mg protein. They increased about 100-fold after 45 min incubation with iron/ascorbate (20 pmol/mg protein) (Table I).

DISCUSSION

In this study, we measured HETEs (5-, 8–12- and 15-isomers) and isoprostanes (8-iso-$PGF_{2\alpha}$ 9α,11α-$PGF_{2\alpha}$ and 9α,11β-$PGF_{2\alpha}$) by GC–MS/NICI in isolated functionally intact rat brain mitochondria and studied their formation during oxidation by iron/ascorbate. Both techniques are capable of detecting these lipid peroxidation products of AA at the fmol level. HETEs and isoprostanes have been

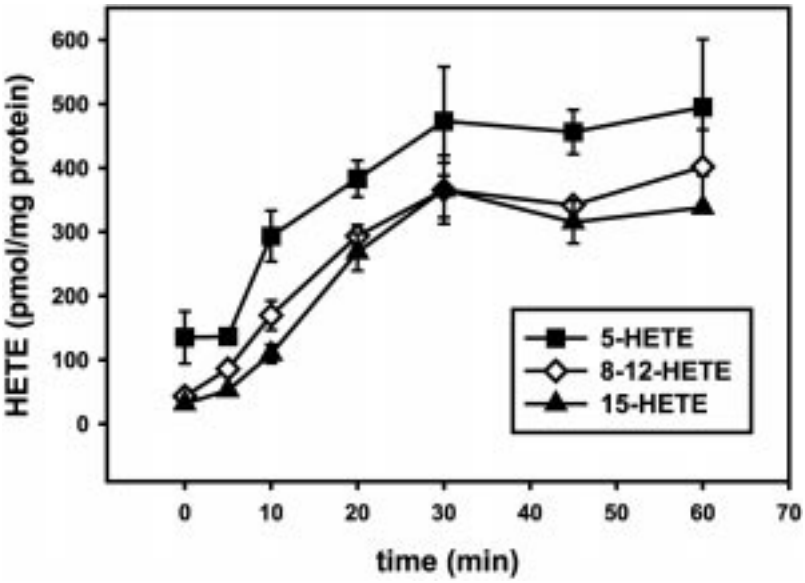

FIGURE 4 Time course of HETEs during iron/ascorbate induced peroxidation in rat brain mitochondria (values are means ± SE of four experiments).

TABLE I Comparison of HETE and isoprostane formation during iron/ascorbate induced oxidative stress. Values "after peroxidation" represent plateau levels after 30 (HETEs) and 60 min peroxidation (isoprostanes) (values are means ± SE of four experiments).

	Before peroxidation (pmol/mg protein)	After peroxidation (pmol/mg protein)	Increase (-fold)
5-HETE	135 ± 41	456 ± 35	3.4
8–12-HETE	52 ± 12	435 ± 62	8.4
15-HETE	31 ± 3	338 ± 3	10.9
8-iso-$PGF_{2\alpha}$	0.21 ± 0.1	20.0 ± 2.0	95.2
9α,11β-$PGF_{2\alpha}$	2.5 ± 0.5	14.3 ± 1.8	5.7
9α,11α-$PGF_{2\alpha}$	5.4 ± 0.7	36.4 ± 5.3	6.7

TABLE II Presence of free and esterified HETEs in rat brain mitochondria during peroxidation (values are means ± SE of four experiments).

	30 min iron/ascorbate induced lipid peroxidation	
	Free HETE (%)	Esterified HETE (%)
5-HETE	28.9 ± 4.6	71.1 ± 3.5
8–12-HETE	2.0 ± 0.8	98.0 ± 5.1
15-HETE	3.2 ± 0.1	96.8 ± 14.2

analyzed formerly in brain homogenates and slices by other authors,[12,33] but we could demonstrate for the first time that freshly isolated rat brain mitochondria had quantifiable amounts of these compounds (220 and 0.21 pmol/mg protein, respectively). Hence, it may be assumed that at least the HETEs are partly of mitochondrial origin.

The ratio of esterified to free (non-esterified) HETEs before peroxidation was 1.1, 7.9 and 5.2 for 5-, 8–12- and 15-HETE, respectively. Interestingly, 5-HETE levels contribute with a higher share to the sum of all HETE isomers and occur less esterified than the other components. Chiral phase high-performance liquid chromatography of HETE isomers (5- and 15-HETE) indicated almost equal amounts of S- and R-HETEs suggesting that the majority of these lipid peroxidation products are formed via non-enzymatic oxidation reactions (H. Kühn, D. Hirsch, I. Wiswedel, unpublished data).

Arachidonic acid is highly susceptible to peroxidation *in vitro* because of the number of double bonds in the molecule.[34] Therefore, it is important to minimize artifact formation during storage and/or during sample processing. In the present study, the following precautions were taken: (1) Brain tissue samples were immediately frozen in liquid nitrogen and kept at −80°C after tissue collection. Lyras *et al.*[35] presented the evidence that post-mortem lipid peroxidation does not occur in brain samples under such conditions; (2) BHT, a chain-breaking antioxidant, was added to the samples before homogenization at a level which prevented brain peroxidation as measured by other assays; and (3) the samples were kept on ice during preparation of mitochondria.

Therefore, our data suggest that HETEs (5-, 8–12- and 15-isomers) and F_2-isoprostanes in freshly isolated rat brain mitochondria are formed *in vivo*, presumably by free radical attack on AA residues

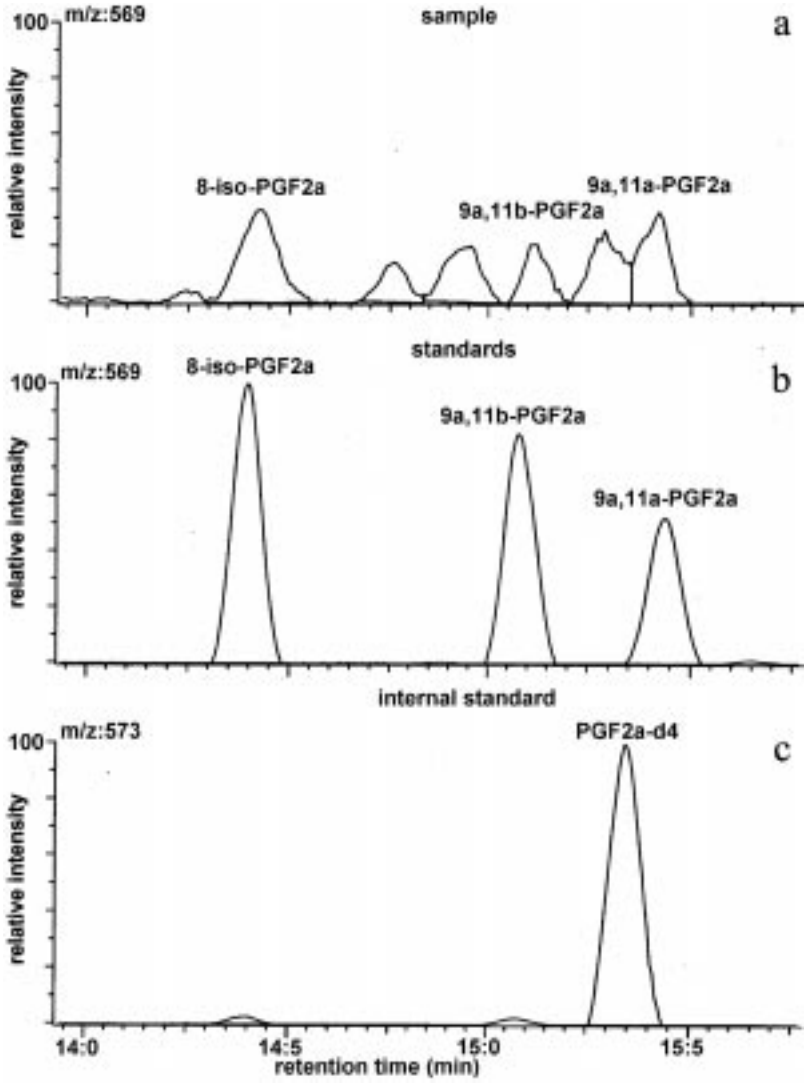

FIGURE 5 Representative GC–MS / NICI chromatogram showing a typical pattern of F_2-isoprostanes in iron / ascorbate treated rat brain mitochondria: (a) oxidized brain mitochondria; (b) authentic PGF_2-like compounds; and (c) $PGF_{2\alpha}$-d_4 as the internal standard. The signal at m / z 569 represents PGF_2-like compounds and that at m / z 573 represents the tetradeuterated internal standard.

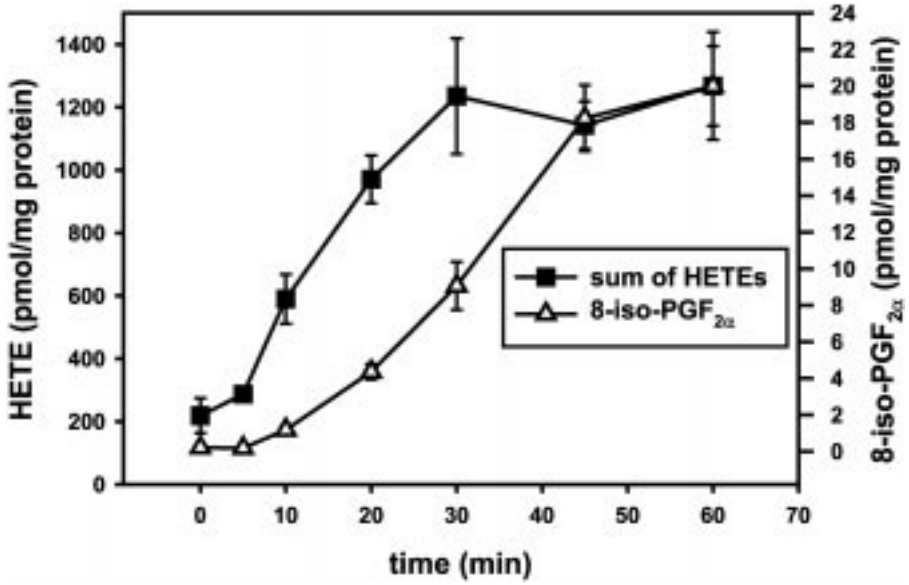

FIGURE 6 Time course of formation of 8-iso-$PGF_{2\alpha}$ and sum of hydroxyeicosatetraenoic acids (5-, 8–12- and 15-HETE) during iron / ascorbate induced oxidative stress in rat brain mitochondria (values are means ± SE of three experiments).

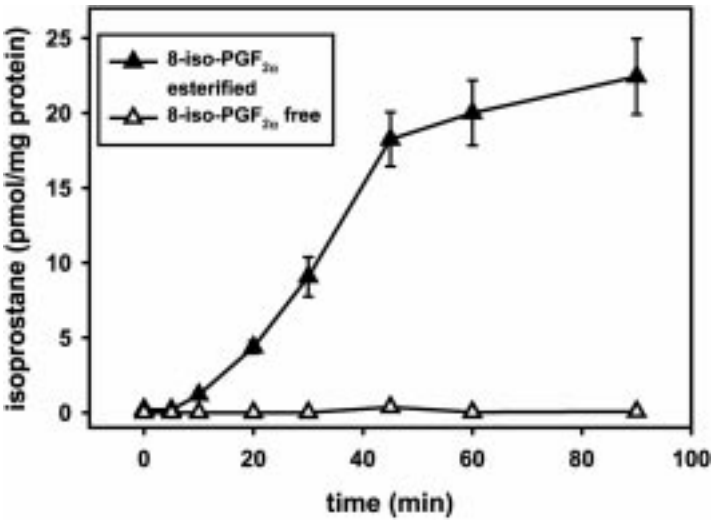

FIGURE 7 Formation of free (non-esterified) and esterified 8-iso-$PGF_{2\alpha}$ during the time course of iron / ascorbate induced peroxidation of rat brain mitochondria (values are means ± SE of four experiments).

esterified into phospholipids, since these lipid peroxidation products were predominantly present in the esterified form. This finding is known for isoprostanes and HETEs from different reports.[10,12,36,37] Oxidative modification of AA and the resulting formation of HETEs and isoprostanes within phospholipids might affect mitochondrial functions by changing the biophysical properties of the membrane, e.g. degree of fluidity. Additionally, specific biological activities may be exerted by HETEs and isoprostanes bound to the phospholipids. Otherwise the lipid mediators can be liberated by phospholipases (preferentially phospholipase A_2) which are known to be activated under conditions of oxidative stress[38] and exerting their actions as free metabolites.

Exposure of the brain mitochondria to iron / ascorbate as the oxidizing system resulted in a time-dependent accumulation of HETEs and 8-iso-$PGF_{2\alpha}$ as well as other isoprostanes. Accumulation of HETEs occurred gradually with no lag-phase; the reaction reached a maximum at 30 min. The formation of HETEs coincided with accumulation of TBARS and depletion of AA as well as the lipid-soluble antioxidant α-tocopherol. In the case of 8-iso-$PGF_{2\alpha}$ formation, there was a 10 min delay period before product accumulation. The formation of 8-iso-$PGF_{2\alpha}$ reached a maximum level at about 45 min. Our data suggest that 8-iso-$PGF_{2\alpha}$ represents a relatively late marker, not only in relation to HETEs and TBARS, but also to the fast exhaustion of AA and α-tocopherol. It should be noticed that the endogenous α-tocopherol levels in isolated functionally intact rat brain mitochondria reported here, are about seven times higher than those described for isolated rat liver mitochondria.[31] The exhaustion of antioxidants (glutathione, α-tocopherol and ubiquinol) and the progressive loss of mitochondrial functions (respiratory rate, enzymes of the respiratory chain, membrane potential) during the lag-phase of iron / ascorbate induced peroxidation were reported some years ago for isolated rat liver mitochondria,[15,31,39,40] but no such information is available for rat brain mitochondria.

Another important finding from this study is that basal levels of HETEs were increased only 6-fold after 30 min incubation with iron / ascorbate while that for the 8-iso-$PGF_{2\alpha}$ was 100-fold. One possible explanation for the difference in the rate of accumulation of HETEs and 8-iso-$PGF_{2\alpha}$ during the severe oxidative stress is that the enhanced formation of HpETEs exceeds the capacity to reduce them, e.g. by the mitochondrial form of phospholipid hydroperoxide glutathione peroxidase[41] and thus favoring the formation of other lipid peroxidation products including isoprostanes. Otherwise isoprostanes are considered as chemically stable end products of lipid peroxidation.[42]

These findings suggest the assessment of neither HETEs nor 8-iso-$PGF_{2\alpha}$ by itself, but measurement of both simultaneously, is adequate to study the role of oxidative stress *in vitro* and furthermore in certain disorders or diseases.

In conclusion, we have introduced two highly specific and sensitive techniques that should be of value in investigating AA peroxidation, and illustrated its application using rat brain mitochondria. The formation of HETEs and isoprostanes increases highly in the model of iron / ascorbate mediated injury and is related to the antioxidant status and the degradation of AA. It must be mentioned, however, that HETEs and isoprostanes do not represent major products of non-enzymatic AA oxidation in rat brain mitochondria exposed to iron and ascorbate. Secondary decomposition of AA may be the prevailing overall reaction. Nevertheless, the improved

GC–MS techniques are of great importance to assess oxidative stress status in many different areas, especially in human diseases.[28,43,44]

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