The Biochemistry of the Isoprostane, Neuroprostane, and Isofuran Pathways of Lipid Peroxidation

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Isoprostanes are prostaglandin-like compounds that are formed non-enzymatically by free radical-catalyzed peroxidation of arachidonic acid (C20:4ω6). Intermediates in the pathway of the formation of isoprostanes are labile prostaglandin H_2-like bicyclic endoperoxides (H_2-isoprostanes). H_2-isoprostanes are reduced to form F-ring isoprostanes (F_2-isoprostanes), but they also undergo chemical rearrangement in vivo to form E_2- and D_2-isoprostanes, isothromboxanes, and highly reactive acyclic γ-ketoaldehdyes (isoketals). E_2- and D_2-isoprostanes also undergo dehydration in vivo to form cyclopentenone A_2- and J_2-isoprostanes. Docosahexaenoic acid (C22:6ω3) is highly enriched in neurons in the brain and is highly susceptible to oxidation. Free radical-catalyzed oxidation of docosahexaenoic acid results in the formation of isoprostane-like compounds (neuroprostanes). F_4-, D_4-, E_4-, A_4-, and J_4-neuroprostanes and neuroketals have all been shown to be produced in vivo. In addition, we recently discovered a new pathway of lipid peroxidation that forms compounds with a substituted tetrahydrofuran ring (isofurans). Oxygen concentration differentially modulates the formation of isoprostanes and isofurans. As oxygen concentrations increase, the formation of isofurans is favored whereas the formation of isoprostanes becomes disfavored.

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ISOPROSTANE PATHWAY OF LIPID PEROXIDATION

In 1975, two interesting reports by 2 groups were published back to back in the same journal showing that prostaglandin (PG)-like compounds could be formed in vitro during autooxidation of linolenic acid (27, 28). However, the possibility that this same chemistry might also occur in vivo was never pursued further. Pursuant to this we reported in 1990 that a series of PGF_2-like compounds are generated by autooxidation during storage of human plasma at -20°C for several months (22), raising concerns about the reliability of measurements of prostanoids in stored biological samples. Subsequently, we established that these same PGF_2-like compounds are formed in vivo by a non-enzymatic free radical-mediated mechanism (23). Because these compounds are isomeric to PGs and have an F-type cyclopentane (prostane) ring, we termed these compounds F_2-isoprostanes (F_2-IsoPs).

The mechanism by which F_2-IsoPs are formed is analogous to the formation of PGs by the cyclooxygenases; PGH_2-like bicyclic endoperoxide intermediates (H_2-IsoPs) are formed which are then reduced to F-ring IsoPs (Figure 1). Three arachidonoyl radicals give rise to the formation of 4 F_2-IsoP regioisomers, each of which is comprised of 8 racemic diastereomers for a total of 64 compounds (Figure 2). We have devised a nomenclature system for IsoPs that has been approved by the Eicosanoid Nomenclature Committee, which is sanctioned by JCBN of IUPAC (37). This nomenclature designates the 4 regioisomer classes according to the carbon number on which the side chain hydroxyl group is attached with the carboxyl carbon being *1*, as indicated in Figure 2.

An important aspect of our discovery is that measurement of F_2-IsoPs has emerged as one of the most reliable approaches to assessing oxidative stress status in vivo (16, 17). A unique aspect of the formation of F_2-IsoPs is that IsoPs are initially formed in situ esterified to phospholipids and subsequently released in free form by phospholipase action (20). Measurement of free F_2-IsoPs, eg, circulating in plasma, can provide an assessment of total body IsoP production, whereas measurement of F_2-IsoPs esterified in tissues can localize and quantify oxidative stress in tissues of interest. Circulating F_2-IsoPs are filtered in the kidney and appear in the urine. Thus, measurement of

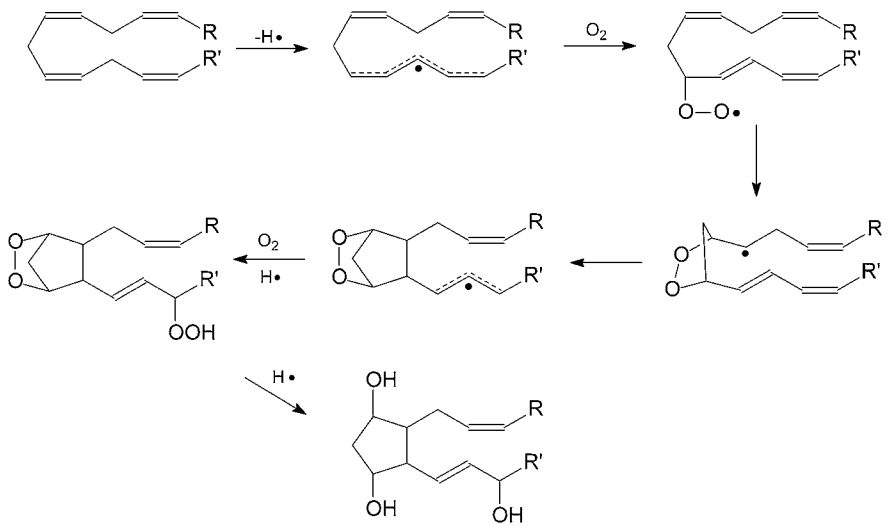

Figure 1. Non-enzymatic free radical mechanism for the formation of F_2-IsoPs.

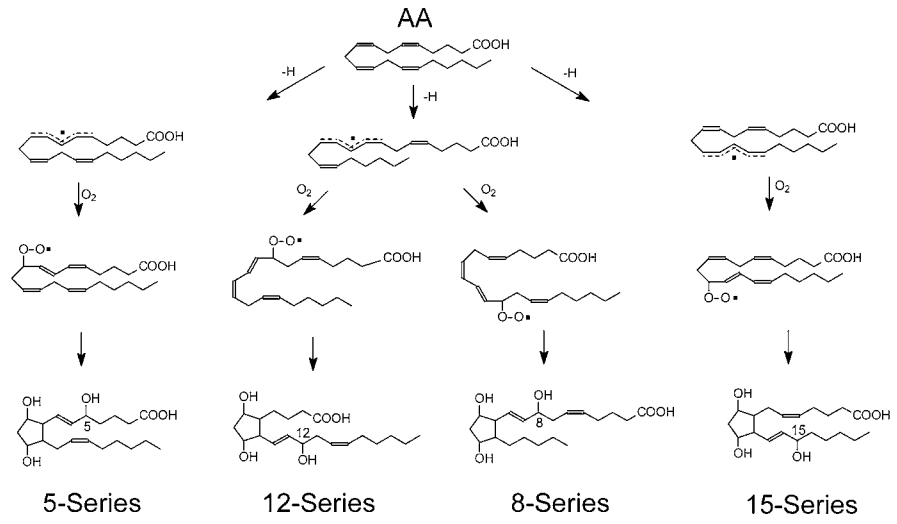

Figure 2. F_2-IsoP regioisomers that are formed.

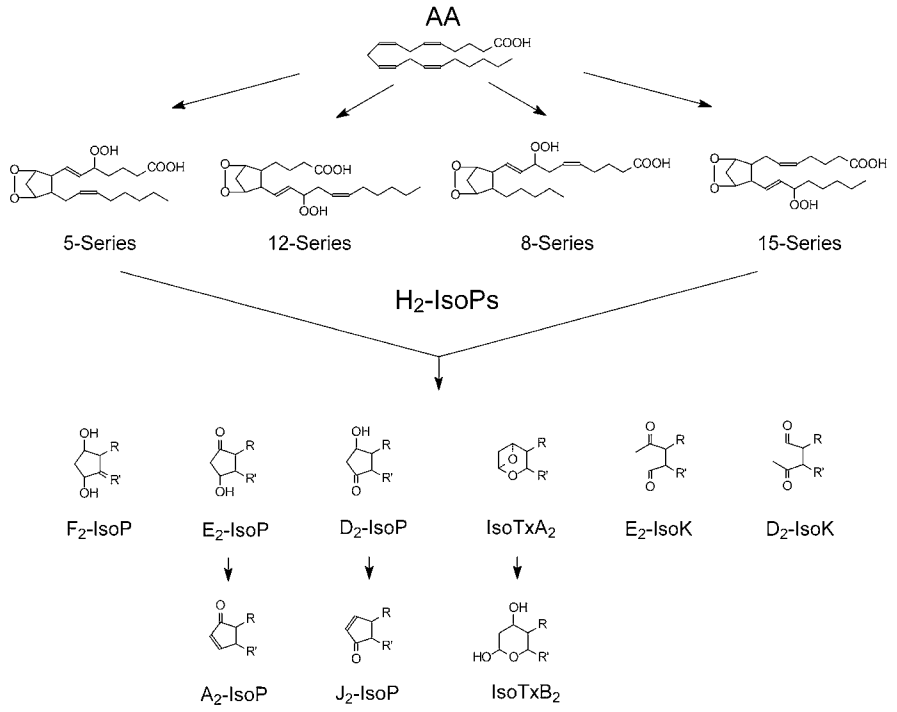

Figure 3. Complete array of products formed in vivo by the IsoP pathway of lipid peroxidation.

urinary F_2-IsoPs also can be utilized to assess total endogenous oxidative stress. However, if there is some kidney disease present that involves oxidative damage, F_2-IsoPs formed in the kidney are directly excreted into urine thereby clouding interpretation of elevated levels of F_2-IsoPs in urine. To circumvent this potential problem, we have identified and developed a GC/MS assay for measurement of the major urinary metabolite of 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$), 2,3-dinor-5,6-dihyro-15-F_{2t}-IsoP which cannot be made in the kidney (32).

Another important aspect of this discovery is that we and others have shown that IsoPs also exert potent biological activities and therefore likely participate as mediators of oxidative injury (23). The complete spectrum of the biological actions of IsoPs will not be detailed in this review. However, we would like to mention biological actions of F_2-IsoPs relevant to brain pathology. We have synthesized 10 F_2-IsoPs. Five of these compounds are 15-series, 2 are 12-series, and 3 are 5-series compounds. All but 2 of the 5-series compounds were very potent vasoconstrictors in brain microvasculature with EC_{50}s ranging from 18.5 to 54.8 nM (15). We also demonstrated that F_2-IsoPs exert their action in this vascular bed by a novel mechanism, by inducing the formation of thromboxane in the endothelium, which in turn contracts the vascular smooth muscle and also causes endothelial cell death (18). Moreover, thromboxane mimetics also induce the generation of F_2-IsoPs thus representing a positive feedback loop resulting in vascular damage.

H_2-IsoP intermediates are highly labile compounds that have a $t_{½}$ of only ~5 minutes in aqueous buffer (13). Therefore, we questioned how efficient the reduction of H_2-IsoPs to F_2-IsoPs is in vivo. If this reduction is not highly efficient then rearrangement products of H_2-IsoPs may also form in vivo (Figure 3). We could not find any evidence for enzymatic reduction of H_2-IsoPs in vivo, but we did obtain evidence that glutathione and other thiols catalyze this reduction (25). PGH_2 undergoes rearrangement to form PGE_2 and PGD_2 in aqueous buffer, so we explored whether H_2-IsoPs undergo rearrangement to form E_2/D_2-IsoPs (Figure 3). Substantial amounts of E_2/D_2-IsoPs were found to be formed both in vitro and in vivo, indicating that the reduction of H_2-IsoPs in vivo is not entirely efficient (24). During oxidation of arachidonic acid in vitro in the absence of reducing agents, eg, thiols, the amounts of E_2/D_2-IsoPs formed greatly exceed the amounts F_2-IsoPs formed; however, in vivo the amounts of F_2-IsoPs formed are greater than E_2/D_2-IsoPs. The same regioisomers of E_2-IsoPs and D_2-IsoPs are formed as with F_2-IsoPs shown in Figure 2.

PGH_2 has also been shown to undergo rearrangement to form small amounts of thromboxane (Tx) so we explored whether H_2-IsoPs also undergo rearrangement to form IsoTxs (Figure 3). Accordingly, we were able to demonstrate the formation of $IsoTxB_2$ compounds both in vitro and in vivo (21). The rearrangement of PGH_2 to Tx involves multiple complex steps so it would be anticipated that the amounts of $IsoTxB_2$ compounds formed would be less than the amounts of E_2/D_2-IsoPs formed, which is what we observed; following treatment of rats with CCl_4 to induce an oxidant injury to the liver the amounts of F_2-IsoPs, E_2/D_2-IsoPs, and $IsoTxB_2$s in the liver were 672 ± 179, 161 ± 37, and 102 ± 30 ng/g

liver, respectively. Again, the same regioisomers of IsoTxs are formed as with IsoPs shown in Figure 2.

Dehydration of PGE_2 and PGD_2 forms cyclopentenone(CP) PGA_2 and PGJ_2, respectively. Therefore, we questioned whether A-ring and J-ring IsoPs may also be formed (Figure 3). Cyclopentenone PGs are reactive compounds owing to the fact that they are α,β unsaturated carbonyls. Considerable work has focused on defining the biological actions of these compounds, in spite of the fact that it remains unclear whether these compounds are actually formed in vivo, which has been an ongoing debate for over 2 decades (3, 19). A study was reported in 1998 describing the detection of Δ^{12}-PGJ_2 in human urine (14). Evidence was provided that Δ^{12}-PGJ_2 was not formed ex vivo by dehydration of PGD_2, although these investigators did not exclude the possibility that PGD_2 had undergone dehydration in the bladder which can be facilitated by protein (12). We had previously shown that PGJ_2 rapidly undergoes Michael addition with glutathione (GSH) in vitro and that conjugation to glutathione in cells appears to be remarkably efficient (1, 2). The fact that PGJ_2 conjugates very rapidly with glutathione may explain why it has been difficult to demonstrate convincingly the formation of PGJ_2 and PGA_2 in vivo by attempting to measure unconjugated compounds. However, since IsoPs are initially formed esterified in membrane phospholipids we reasoned that we should be able to detect the formation of esterified CP IsoPs in tissues because until they are hydrolyzed from phospholipids they would be shielded in the membrane from GSH and other cytoplasmic thiols. Indeed, we were able to detect measurable amounts of A_2/J_2-IsoPs in normal rat liver and levels increased dramatically in the liver of rats treated with CCl_4 to induce an oxidant injury (8). In this model of oxidant injury, F_2-IsoPs, E_2/D_2-IsoPs, and isoTxB_2s levels are also increased greatly in the circulation. However, even using this severe model of oxidative injury we were not able to detect unconjugated A_2/J_2-IsoPs in plasma. This would be consistent with the notion that once they are released from membrane phospholipids CP IsoPs readily undergo conjugation with thiols.

PGH_2 has also been shown to undergo rearrangement to form acyclic γ-ketoaldehydes termed levuglandins (34). These are extremely reactive molecules that rapidly adduct to lysine residues on proteins. Therefore, we undertook studies to explore whether analogous γ-ketoaldehydes, termed isoketals (IsoKs), are also formed as products of the IsoP pathway (Figure 3). As with other IsoPs, 4 regioisomers of both E_2- and D_2-IsoKs are formed. IsoKs were readily detected during oxidation of arachidonic acid in vitro at levels that were intermediate between levels of F_2-IsoPs and E_2/D_2-IsoPs (7). However, we could not detect these compounds during oxidation of biological samples that contain protein. This was subsequently explained by our finding that IsoKs adduct to proteins with extreme rapidity. Therefore, we defined the chemistry of adduction of IsoKs to lysine residues. IsoKs initially form a reversible Schiff base which then proceeds through a pyrrole which becomes oxidized to form lactam and hydroxylactam adducts (5, 7). Oxidation of the pyrrole intermediate also leads to facile cross-linking of proteins. We have developed a method to measure IsoKs in tissues by enzymatically digesting tissue proteins to individual amino acids and quantifying IsoK lysyl adducts by LC/MS/MS using an internal standard formed by adducting 15-E_{2t}-IsoK to [$^{13}C_6$] lysine. Using this approach we were able to demonstrate the formation of IsoK adducts in abundance in vivo in livers from rats treated with CCl_4. Interestingly, owing to their remarkable reactivity, we also demonstrated that IsoKs were adducted to adjacent membrane proteins while still esterified to phospholipids (6). To facilitate our ability to both detect and localize IsoK proteins adducts in tissues by immunohistochemistry, we have developed and validated a single chain antibody (ScFv) that specifically recognizes IsoK lysyl adducts on proteins independent of amino acid sequence (9).

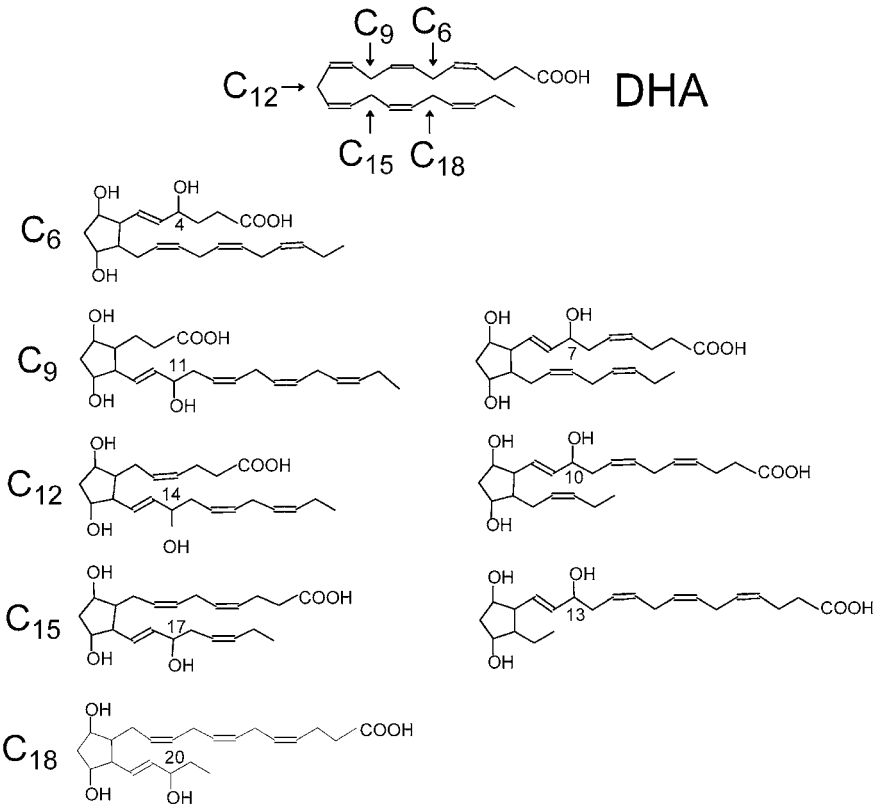

Figure 4. Regioisomers of F_4-NPs formed. Carbon numbers indicate from which hydrogen abstraction occurs to result in specific regioisomer formation.

In summary, Figure 3 demonstrates all of the products we have identified that are formed in the IsoP pathway of lipid peroxidation. We have been able to show that all of the products from the cyclooxygenase pathway are also produced in the IsoP pathway with the exception of prostacyclin (PGI_2). We have attempted to detect the formation of the hydrolysis product of PGI_2, 6-keto-$PGF_{1\alpha}$, during oxidation of arachidonic acid in vitro, but have not

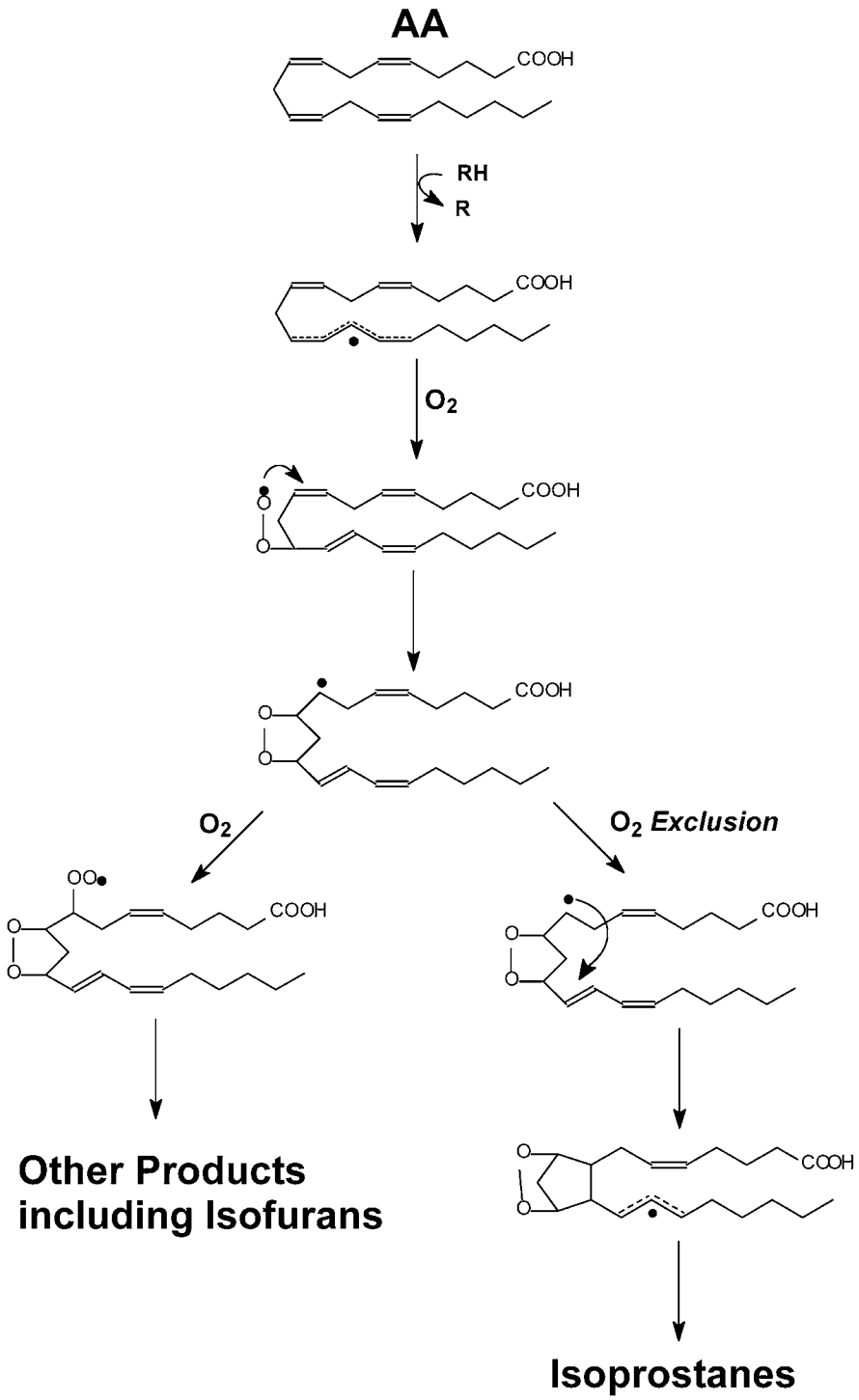

Figure 5. Figure illustrating an "oxygen exclusion" step in the pathway leading to IsoP formation. If oxygen is not excluded, the intermediate carbon-centered radical is attacked by O_2, leading to the formation of other products.

found detectable quantities with lower detection limits of low pg quantities.

NEUROPROSTANE PATHWAY OF LIPID PEROXIDATION

We have also explored whether compounds analogous to IsoPs are also formed during oxidation of other unsaturated fatty acids, in particular docosahexaenoic acid (DHA) (C22:6ω6). Others have shown that F_3-IsoPs are formed during oxidation of eicospentaenoic acid (EPA) (C20:5ω3) (26). However, levels of EPA in humans are very low in the absence of fish oil supplementation. In contrast, DHA is highly enriched in normal human brain and neurons comprising ~25% to 35% of the total fatty acids in aminophospholipids (33, 36). DHA is also more susceptible than AA to oxidation owing to the fact that it contains a greater number of double bonds. Therefore, we reasoned that measurement of IsoP-like compounds formed from oxidation of DHA, which we termed neuroprostanes (NPs), might provide a more sensitive biomarker of neuronal oxidant injury than IsoPs.

Analogous to the IsoP pathway, F-ring NPs would be formed by reduction of H-ring intermediates. However because of the larger number of double bonds in DHA, 8 rather than 4 regioisomers would be formed as a result of abstraction of a bis-allylic hydrogen atom from C_6, C_9, C_{12}, C_{15}, and C_{18}, as shown in Figure 4. This gives rise to 8 NP regioisomers (4-, 7-, 10-, 11-, 13-, 14-, 17-, and 20-series F_4-NPs). We were able to show that F_4-NPs are formed both during oxidation of DHA in vitro and in normal brain tissue (31). Moreover, these initial studies also demonstrated that measurable levels of F_4-NPs were present in CSF from normal individuals and that levels were higher in patients with Alzheimer disease (AD). Further studies supported the notion that measurement of F_4-NPs provided a more sensitive biomarker of oxidative neuronal injury compared to F_2-IsoPs (29).

We have also been able to show that the same array of E_4- and D_4-NPs are formed during oxidation of arachidonic acid in vitro and that levels of these NPs were present in detectable quantities in both rat and human brain (30). In brain, levels of E_4/D_4-NPs are present in about one-third the amount of F_4-NPs. We have not explored whether Tx-ring compounds are formed during oxidation of DHA, although it is highly likely that they are formed, since measuring these compounds would not offer an advantage over measuring F_4-NPs to assess oxidative injury in the brain. At present, it remains to be determined whether NPs exert biological activity.

More recently, we also demonstrated the formation of IsoK-like compounds (neuroketals, NKs) during oxidation of DHA in vitro and also showed that the amounts of NKs formed was ~4-fold greater than the amounts of IsoKs formed (4). We also found the presence of NK protein adducts in normal human brain at a level of ~10 ng/g brain. Interestingly, we have recently

found that the levels of NK adducts measured by LC/MS/MS in hippocampus from brains of patients with AD were significantly increased compared to hippocampus from age-matched controls and that there was intense immunostaining of virtually every neuron in the hippocampus from patients with AD using our single chain antibody against IsoK adducts, which also recognizes NK adducts, and that this was absent in aged-matched controls (manuscript in preparation). These new findings provide the framework to further explore whether NKs are involved in protein aggregation and cross-linking which is a feature of AD.

ISOFURAN PATHWAY OF LIPID PEROXIDATION

The value of F_2-IsoPs as a biomarker of oxidative stress is well appreciated; this has recently been validated in a study comparing many approaches to assess oxidative stress status in vivo (16, 17). Nonetheless, we have recently recognized a shortcoming of this approach when applied to situations involving oxidative injury in settings of elevated oxygen tension. In the IsoP pathway, there is a carbon-centered radical that undergoes intra-molecular attack to form the cyclopentane ring. This is an "oxygen exclusion" step because O_2 competes with this attack of the carbon-centered radical. If the carbon-centered radical is attacked by O_2, "other products" than IsoPs are formed (Figure 5). Thus we hypothesized that as oxygen tension increases, the formation of IsoPs becomes disfavored and the formation of "other products" would become favored. Recently we reported the discovery of new products of lipid peroxidation that are formed as a result of oxygen attack on that carbon centered radical (11). These compounds have a substituted tetrahydrofuran ring, hence we have termed them isofurans (IsoFs). It was determined that 2 mechanisms are involved in the formation of IsoFs; labeling studies using $^{18}O_2$ and $H_2{}^{18}O$ showed that some IsoFs incorporate all 4 oxygen atoms from molecular oxygen whereas others incorporate 3 oxygen atoms from molecular oxygen and one oxygen atom from H_2O. There are 2 mechanisms proposed for the formation of IsoFs: the cyclic peroxide cleavage pathway and the epoxide hydrolysis pathway. A total of 8 regioisomers are formed that are comprised of 16 racemic diastereomers for a total of 256 compounds.

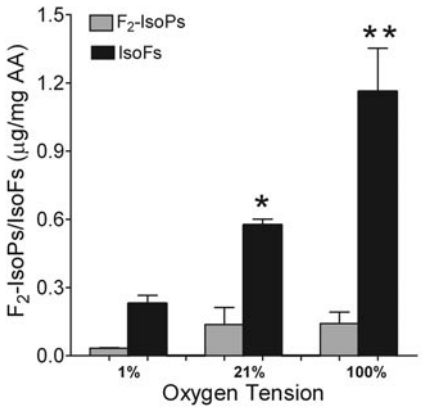

Figure 6.

Consistent with our hypothesis regarding the effect of oxygen tension on the formation of IsoF and IsoPs, we found that as oxygen was increased from 1% to 21% there was only a modest increase in the formation of F_2-IsoPs during oxidation of arachidonic acid in vitro which did not increase further upon increasing oxygen tension to 100%. In contrast, there was a significant increase in the formation of IsoFs when oxygen was increased from 1% to 21% and a further robust increase when oxygen tension was further increased to 100% (Figure 6). We were also able to demonstrate this same phenomenon in vivo; levels of IsoFs esterified in lungs of mice exposed to 100% O_2 were significantly increased compared to mice breathing 21% oxygen whereas levels of F_2-IsoPs were not different.

These findings may also be applicable to other situations such as studies involving the occurrence of oxidative stress in cultured neurons and other cells that are exposed to non-physiologically high concentrations of O_2 under culture conditions in vitro. Another relevant situation would be studies of diseases associated with oxidative stress and mitochondrial dysfunction. Cellular oxygen tension theoretically would be increased if mitochondria are dysfunctional due to diminished consumption of O_2 by the mitochondria. We have obtained support for this hypothesis by measuring both F_2-IsoPs and IsoFs in the substantia nigra from patients with Parkinson disease. Mitochondrial dysfunction is a defining feature of this disease (35). We found that IsoFs, but not F_2-IsoPs, were significantly increased in the substantia nigra of patients with Parkinson disease compared to aged-matched controls (10). We are currently in the process of characterizing the formation of neurofurans in vitro and in vivo.

Based on these observations, we feel that combined measurements of both F_2-IsoPs and IsoFs by GC/MS offer the most reliable approach to assess oxidative stress status in vivo. Simultaneous measurement of both F_2-IsoPs and IsoFs is greatly facilitated by the fact that these 2 compounds co-purify through the assay we developed for F_2-IsoPs, while their masses differ by 16 Da, allowing simultaneous monitoring of the ion channel for F_2-IsoPs and the ion channel 16 Da higher for IsoFs.

SUMMARY

A wide array of compounds are formed as products of both the IsoP and NP pathways of free radical mediated lipid peroxidation. Measurement of F_2-IsoPs and F_4-NPs has proven of considerable value in assessing oxidative stress status in vivo in systemic biological fluids and tissues for F_2-IsoPs and in the brain and cerebral spinal fluids for F_4-NPs. Moreover, a number of F_2-IsoPs exert potent biological actions in brain vasculature and other vascular beds. In addition, the chemically reactive cyclopentenone IsoPs and NPs, as well as IsoKs and NKs exert potentially important biological actions owing to their reactivity. While great advances have been made in the research related to IsoPs and NPs, many additional avenues for future investigation remain to be explored.

ACKNOWLEDGMENTS

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