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Forum Review

Isofurans: Novel Products of Lipid Peroxidation that Define the Occurrence of Oxidant Injury in Settings of Elevated Oxygen Tension

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ABSTRACT

We recently reported the discovery of isofurans, novel products of free radical-induced peroxidation of arachidonic acid that exhibit favored formation with increasing oxygen concentrations. In this review, the biochemistry of isofuran formation is compared with that of isoprostanes, with an emphasis on the mechanistic basis for the favored formation of isofurans at elevated oxygen tensions. In addition, the formation of isofurans in various disease states *in vivo* is also discussed. Parkinson's disease is presented as a disease model involving mitochondrial dysfunction, a situation in which quantification of isofurans can provide a uniquely sensitive indicator of oxidant injury. Measurement of isofurans has also provided unexpected insights into the earliest events in hyperoxic lung injury, an important clinical problem in which measurement of isofurans might prove to be uniquely valuable in the evaluation of approaches to limit this injury. These two settings are then used as models to suggest a variety of other pathological settings in which measurement of isofurans together with isoprostanes could provide a complete and robust picture of oxidative stress status in ongoing and future investigations. *Antioxid. Redox Signal.* 7, 202–209.

INTRODUCTION

OXIDATIVE STRESS refers to the overproduction of free radicals/oxidants and/or an insufficiency of antioxidant/radical-detoxifying systems that results in deleterious modification of biological macromolecules. Oxidative stress continues to be linked to an ever-increasing number of dysfunctional states *in vivo*, of which atherosclerosis, neurodegenerative diseases, cancer, inflammation, and the normal aging process are only a few examples. The deleterious effects of oxidative stress that contribute to disease most frequently manifest as chemical modification of proteins, nucleic acids, and lipids.

Assessment of the role of oxidative stress in the pathogenesis or manifestations of any disease state requires a sensitive and specific *in vivo* marker of oxidant injury. Over the last 14 years, measurement of F_2-isoprostanes (F_2-IsoPs) has emerged as probably the most reliable approach to assess oxidative stress status *in vivo.* The biochemistry of IsoP formation in various *in vitro* and *in vivo* conditions has been extensively studied and is reviewed in a number of other reviews in this Forum issue. However, an important and previously unappreciated limitation of IsoP measurement can be recognized by a detailed examination of the mechanism by which the H_2-IsoP intermediates in the IsoP pathway are formed. This mechanism involves an exocyclization of a carbon-centered radical to form the bicyclic endoperoxide. This reaction, however, is in competition with the addition of a molecule of oxygen to the carbon-centered radical to yield a peroxyl radical, which precludes the formation of IsoPs. Thus, these two reactions are competing and mutually exclusive, and any setting in which the addition of oxygen is favored relative to the exocyclization will result in an underestimation of the extent of lipid peroxidation and oxidant injury using measurements of F_2-IsoP.

In a biological setting of oxidative stress, the ambient oxygen concentration is the most important determinant of the

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rates of these two exclusive competing reactions. As ambient oxygen concentration increases, IsoP formation becomes increasingly disfavored as the formation of other lipid peroxidation products becomes more favored (23). Quantification of one or more of these products in combination with the IsoPs would therefore provide a more complete picture of the extent of oxidant injury across all oxygen concentrations. In this article, we discuss the discovery and characterization of isofurans (IsoFs), unique products of lipid peroxidation whose formation becomes favored with increasing oxygen concentration. We also discuss an ever-growing number of *in vivo* situations where quantification of IsoPs and IsoFs together gives a more accurate assessment of the extent of oxidant injury than that of either product alone.

DISCOVERY AND BIOCHEMISTRY OF ISOFURAN FORMATION

In the course of limited mass scanning of lipid peroxidation products formed during *in vitro* oxidation of arachidonic acid by gas chromatography negative ion chemical ionization mass spectrometry (GC/NICI/MS), a series of compounds was observed that had chromatographic properties quite similar to those of F_2-IsoPs, but that had a mass-to-charge ratio indicating that these compounds had a mass 16 Da greater than F_2-IsoPs. The chromatographic and derivatization conditions used for the isolation and detection of products formed allowed for only a limited number of potential substituents on a lipid backbone that would form compounds that cochromatograph with F_2-IsoPs and were 16 Da higher than F_2-IsoPs. Specifically, the incorporation of an additional atom of oxygen would account for the mass difference and would not dramatically alter the chromatographic properties of the resultant compounds compared with those of F_2-IsoPs.

Initial predictions as to how a single atom of oxygen could be incorporated focused specifically on carbonyl-type functionalities and epoxides. Indeed, the existence of epoxyisoprostanes has been reported in the literature (40). However, these two possibilities were quickly ruled out because the observed compounds failed to react both with strong reducing agents such as sodium borohydride, which would be indicative of the presence of carbonyl functionalities, and with strong acid, which would hydrolyze epoxides. A more general investigation of the functional groups present was then undertaken. Analysis of the compounds as a deuterated trimethylsilyl ether derivative indicated that the observed compounds had three hydroxyl groups. Analysis after catalytic hydrogenation indicated that the compounds contained two double bonds. Taking into account all of these constraints, a structure was proposed that was consistent with all of the available data (Fig. 1). Because the structure proposed contains a substituted tetrahydrofuran ring and a series of isomers were found to be formed, we termed these compounds isofurans (IsoFs) (10). This structure is consistent with a structure elucidated by Pace-Asciak in 1971, although in these studies the compound under investigation (or its immediate precursors) was thought to have been generated enzymatically by the cyclooxygenase and represented only a single compound rather than a series of nonenzymatically generated products (24).

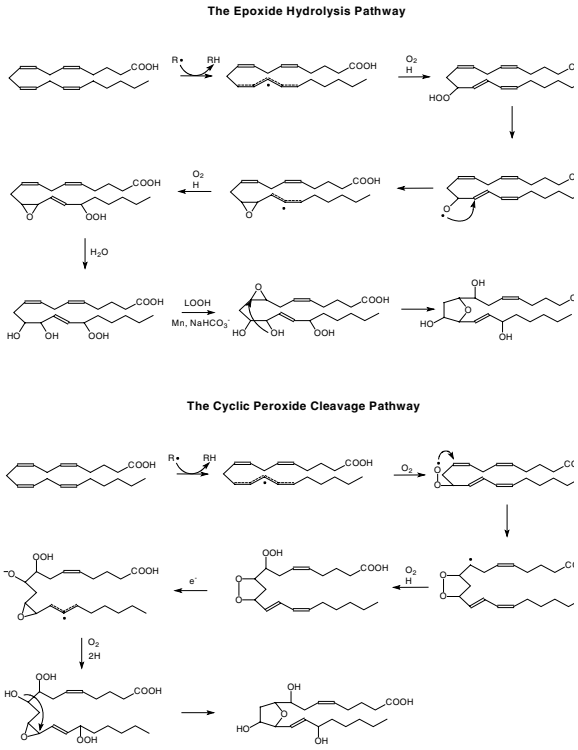

FIG. 1. Two pathways contributing to the formation of IsoFs. The epoxide hydrolysis pathway involves incorporation of one oxygen atom from water into the final product. The cyclic peroxide cleavage pathway incorporates all oxygen atoms from gaseous oxygen. Together, these pathways form eight IsoF regioisomers, one of which is pictured above.

To confirm the presence of an additional oxygen atom to account for the 16-Da difference between IsoFs and F_2-IsoPs, arachidonic acid was oxidized in the presence of $^{18}O_2$ and $H_2{}^{18}O$ and the products analyzed by GC/NICI/MS. These studies not only confirmed the presence of an additional oxygen atom in IsoFs, but also demonstrated that two distinct mechanisms are involved in IsoF formation: one involving the incorporation of a single atom of oxygen from water and three atoms of oxygen from molecular oxygen, and one in which all oxygen atoms are incorporated from molecular oxygen. The two mechanisms proposed for the formation of IsoFs are shown in Fig. 1. These proposed mechanisms allowed for the prediction of the structures of eight IsoF regioisomers, which were subsequently supported by fragmentation data from electron ionization mass spectrometry. Very recently, one of the IsoF regioisomers has been synthesized, and the fragmentation of this synthetic compound is essentially identical to the fragmentation assigned to the same regioisomer formed during *in vitro* oxidation of arachidonic acid (unpublished observations). Moreover, the proposed mechanisms allowed for the prediction that ambient oxygen concentration would have differing effects on IsoF and F_2-IsoP formation. The mechanistic basis for this prediction is summarized in Fig. 2, which depicts two mutually exclusive competing reactions for which oxygen concentration is the critical determinant. As oxygen concentration increases, F_2-IsoP formation

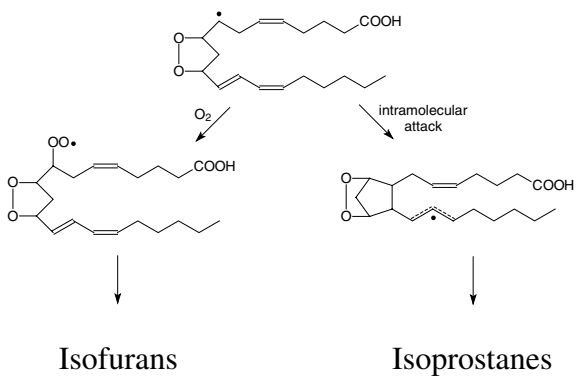

FIG. 2. Hypothesized mechanistic basis for the differential effects of oxygen on IsoF and IsoP formation. From a common intermediate for both IsoF and IsoP formation, two mutually exclusive and competing reactions are hypothesized, with oxygen concentration as the important rate determinant. Higher oxygen concentration favors IsoF formation and disfavors the intramolecular attack by the carbon-centered radical pictured that is necessary to form IsoPs.

was predicted to become increasingly disfavored as IsoF formation was predicted to become more favored. Previous data from our laboratory indicated that formation of IsoPs *in vitro* plateaus at 21% oxygen (23). *In vitro* oxidation of arachidonic acid under varying oxygen concentrations again confirmed that F_2-IsoP formation plateaus at 21% oxygen. In contrast, however, IsoF formation continued to increase up to 100% oxygen (Fig. 3), in keeping with predictions from the proposed mechanisms.

More importantly, IsoFs were then detected *in vivo* and were shown to reflect differences in ambient oxygen concentrations in tissues. These data are summarized in Fig. 4. In a tissue with a very low oxygen tension, the liver, the formation

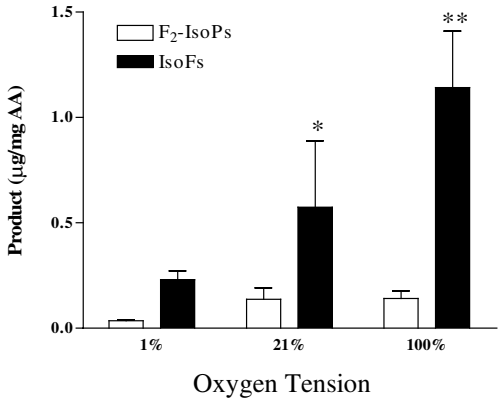

FIG. 3. Effect of oxygen tension on F_2-IsoP and IsoF formation during oxidation of arachidonic acid (AA) *in vitro*. Each column represents the mean ± SEM for three independent experiments. Arachidonate was oxidized using an Fe/ADP/ascorbate system, and all solutions were quantitatively degassed and then replaced with the appropriate oxygen concentration and nitrogen balance. *$p < 0.05$, **$p < 0.001$ versus all other groups by ANOVA.

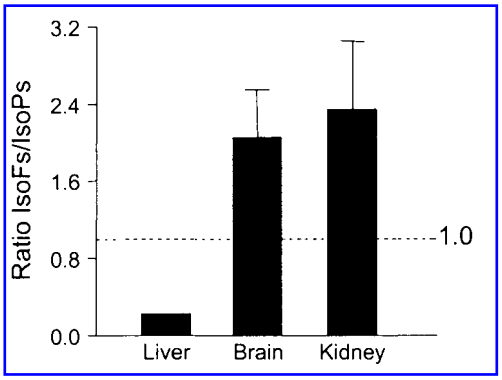

FIG. 4. Ratio of IsoFs to F_2-IsoPs in different tissues. Ratios of IsoF/F_2-IsoP levels were measured in rat liver ($n = 3$), brain ($n = 4$), and kidney ($n = 6$). "Brain" represents the hippocampus, one of the most oxygen-rich regions of the brain. Results are shown as the means ± SEM.

of esterified F_2-IsoPs is highly favored compared with the formation of IsoFs. In contrast, in richly oxygenated tissues like kidney and brain, the level of IsoF formation exceeds that of F_2-IsoPs by greater than twofold. Not only did these observations demonstrate that IsoFs are formed *in vivo* and that their formation *in vivo* is favored over the formation of IsoPs at relatively higher oxygen concentrations, these data also suggested that combined measurements of both F_2-IsoPs and IsoFs may provide a more complete and reliable index of oxidative stress status under all conditions than measurement of either analyte alone. Evaluation of this hypothesis is greatly facilitated by the fact that F_2-IsoPs and IsoFs copurify using the assay for F_2-IsoPs and therefore can be quantified in a single GC/NICI/MS assay as a pentafluorobenzyl ester trimethylsilyl ether derivative by the additional monitoring of the $M-\bullet CH_2C_6F_5$ ion for IsoFs. This potential utility of measuring IsoFs as an index of oxidative stress remains to be fully explored, although the possibilities are great. Moreover, determining the ratio of IsoFs and F_2-IsoPs formed could in theory be used to monitor responses to a wide variety of therapies, such as drugs or procedures to restore blood supply in peripheral vascular disease, synthetic oxygen carriers (so-called "blood substitutes") that are in development to reduce the need for blood products in surgery, and antiangiogenesis measures targeted at disrupting blood supply to solid tumors, to name just a few.

ISOFURANS IN PARKINSON'S DISEASE AND MITOCHONDRIAL DYSFUNCTION

In defining disease settings in which IsoFs would be a useful adjunct to quantification of F_2-IsoPs as an index of oxidant injury, one must define disease states where oxygen concentrations are perturbed and, specifically, where oxygen concentrations are elevated above normal. This can, at first glance, seem like a relatively short list, as many disease states involve decreased oxygen concentrations in tissues, as opposed to increased oxygen concentrations. One pathologic state that seems an obvious choice for investigation with

TABLE 1. DEMOGRAPHIC DATA FOR CONTROLS AND PATIENTS WITH PD, DLB, MSA, AND AD

	Control	*PD*	*DLB*	*MSA*	*AD*
Number	5	7	4	4	5
Age (years)	79.0 ± 4.9	76.4 ± 3.2	69.3 ± 9.2	77.4 ± 8.8	80.6 ± 10.2
Postmortem interval (h)	6.6 ± 3.7	8.6 ± 6.9	11.2 ± 7.8	10.2 ± 10.4	7.8 ± 6.2
Male/female	3/2	4/3	2/2	2/2	2/3

IsoFs is hyperoxia-mediated lung injury, so-called oxygen toxicity (3, 18). This is discussed in detail in the next section. However, a larger group of potential disease states that may not be immediately obvious are those involving mitochondrial dysfunction.

The underpinning hypothesis is that mitochondrial dysfunction creates an environment conducive for IsoF formation in two important respects. First, mitochondrial dysfunction and blockade of the electron transport chain are widely known to enhance greatly free radical leakage from the mitochondria, much of which is thought to be in the form of superoxide anion. Indeed, compounds such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (1, 29, 35) and rotenone (2, 13, 34), both of which are inhibitors of mitochondrial complex I, have been shown to enhance superoxide production and release from mitochondria. Secondly, mitochondrial dysfunction/blockade leads to a decrease in overall oxygen consumption by the mitochondria. As mitochondria are the main consumers of oxygen in most living cells, this should lead to an overall increase in intracellular oxygen concentration. With mitochondrial dysfunction, then, a setting exists involving both increased free radical production and increased intracellular oxygen concentration, which would favor the formation of IsoFs, but disfavor the formation of F_2-IsoPs.

Through collaboration with Dr. Jing Zhang at the University of Washington, we were afforded a unique opportunity to test this hypothesis by examining IsoF and F_2-IsoP levels in substantia nigra (SN) tissue collected post mortem from patients with Parkinson's disease (PD) (11). PD is a disease in which mitochondrial dysfunction, especially involving complex I, has been well documented and a disease in which oxidative stress is thought to play an important role. We examined esterified levels of IsoFs and F_2-IsoPs in SN samples from PD patients and compared them with age-matched controls. In addition, levels were also measured in the SN from brains of other patients with other disorders of neurodegeneration for comparison. Samples of SN from patients with Alzheimer's disease (AD) were included as a regional specificity control, as AD is a neurodegenerative disease that involves oxidative injury to specific areas of the brain (31) while sparing the SN. Patients with multiple system atrophy (MSA) were included to ensure that any differences detected in the PD patients were not simply due to cell death in the SN. MSA involves neuronal loss in many brain regions, including the SN, but oxidative stress has not been strongly implicated in the pathogenesis of MSA. Finally, patients with dementia with Lewy bodies, or diffuse Lewy body disease (DLB), were examined. DLB is thought to be a disease lying near the middle of a spectrum defined by AD at one end and PD at the other. We sought to determine whether DLB pathology in the SN was biochemically more similar to AD or PD, as this could have important implications for the pathogenesis of DLB, which is poorly understood at present.

The demographic data for the patients included in this study are shown in Table 1, and the results of the study are shown in Fig. 5. In keeping with the initial hypothesis, levels of IsoFs esterified in the SN were significantly elevated in PD patients compared with controls, whereas levels of esterified F_2-IsoPs were not. Interestingly, we found that DLB was accompanied by oxidant injury in the SN more similar to PD than to AD. These data, then, may point to a role for mitochondrial dysfunction and oxidative stress in the pathogenesis of DLB that merits further investigation.

These studies also provide considerable support for the value of measuring IsoFs as a biomarker of oxidative stress *in vivo*. First, these studies represent a model of human disease in which IsoFs provide unique information that could not have been obtained by measurement of F_2-IsoPs alone. This greatly supports the notion that measurement of IsoFs and F_2-IsoPs in combination can provide a more accurate index of

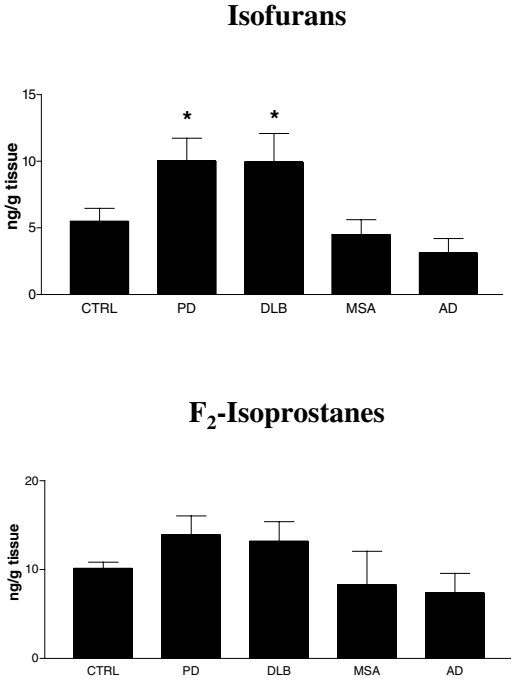

FIG. 5. Analysis of IsoFs and F_2-IsoPs esterified in SN. Measurements are presented as ng of product/g wet weight of tissue. PD and DLB patients show a significant increase in IsoFs compared with all other groups (*$p < 0.05$), whereas F_2-IsoPs show no significant increase for any group in the study.

oxidant injury than measurement of either product alone. Secondly, these studies serve as a "proof of concept" for the notion that IsoFs may be an especially useful biomarker of oxidant injury in settings of mitochondrial dysfunction. This greatly extends the number of potential pathologic states in which IsoFs might prove to be the best indicator for the involvement of oxidative stress. Mitochondrial dysfunction has been observed or hypothesized in a variety of diseases involving mutations of the mitochondrial genome, the so-called familial mitochondriopathies (12, 20, 27). The involvement of oxidative stress in the pathogenesis of these diseases has been only cursorily investigated, and future investigations utilizing measurements of IsoFs could prove to be very informative. A number of drugs that are widely used clinically, most notably drugs used to treat HIV/AIDS as part of highly active antiretroviral therapeutic regimens, show mitochondrial toxicity as an important side effect (4, 22, 39, 42). As with the familial mitochondriopathies, examination of IsoF formation in patients being treated with these drugs might shed light on the involvement of oxidative stress and lipid peroxidation relating to the toxicity profiles of these drugs. Finally, mitochondrial mutations and/or mitochondrial dysfunction have been implicated in the neoplastic and malignant potential of a variety of tumors (6, 8, 19, 25). As with the other examples given above, measurement of IsoFs in these tumors could provide insight into the links that might exist between mitochondrial mutations/dysfunction, oxidative stress, and tumor progression.

ISOFURANS IN SETTINGS OF ABNORMAL OXYGEN DELIVERY

The most obvious situations in which quantification of IsoFs would be expected to provide insight into oxidative damage are those in which oxygen supply is deliberately altered. Most notable is the setting in which lung injury can occur in patients that are ventilated with supraphysiologic concentrations of oxygen, *i.e.,* concentrations of oxygen greater than that in room air (21%). Oxygen toxicity refers to the well recognized clinical problem in which a patient experiences lung damage and pulmonary dysfunction after breathing elevated concentrations of oxygen for extended periods of time. The lung damage and pulmonary dysfunction are characterized by atelectasis, pulmonary edema, neutrophil infiltration, and ultimately a presentation indistinguishable from the acute respiratory distress syndrome. Although the exact concentration of oxygen and duration of exposure required to produce lung damage are not absolutely defined in humans, administration of concentrations of oxygen higher than 60% for limited periods of time (a few hours) is generally considered safe.

Oxygen has been presumed to cause damage to the lung by inducing an oxidant injury to bronchial epithelial and pulmonary endothelial cells. Insight into how high concentrations of oxygen might induce an oxidant stress was elucidated by Freeman and Crapo who demonstrated that production of superoxide anion by lung mitochondria increases with increasing oxygen tension (14). Direct evidence, however, for oxidant injury to epithelial or endothelial cells in oxygen toxicity has been equivocal, with some studies finding increased levels of biomarkers of oxidative injury (7, 21, 30), whereas others have failed to show such increases (16, 36). Cellular responses to hyperoxia are also mixed, with some studies finding up-regulation of antioxidant defense systems (15, 38), whereas others have failed to show this (9, 26). Protection from hyperoxic lung injury by administration of antioxidant compounds (5, 32, 33) or by enhancing defenses against free radical damage (17, 28, 41) has been somewhat effective, although even these findings are not unequivocal (16, 37). Thus, it remains unclear whether oxidant injury plays a fundamental causative role in pulmonary oxygen toxicity.

We had previously explored the potential role of oxidant injury in oxygen toxicity in a sheep by measuring F_2-IsoPs. In that study, we quantified levels of F_2-IsoPs in plasma, urine, bronchoalveolar lavage fluid, and lymph draining from the lung in a sheep breathing >98% oxygen for up to 96 h. Despite apparent marked respiratory distress in the animal, levels of F_2-IsoPs did not increase in any of these fluids. With the current realization that the formation of F_2-IsoPs becomes highly disfavored whereas the formation of IsoFs becomes highly favored in the presence of high concentrations of oxygen, we revisited the question of the role of oxidative stress in oxygen toxicity utilizing measurements of IsoFs in mice exposed to a high concentration of oxygen.

Adult female C57/Bl6 mice were exposed to >98% oxygen, and at various time points the animals were killed and the lungs removed for quantification of esterified IsoFs and F_2-IsoPs. During a brief time course extending over 3–4 h, F_2-IsoP levels in the lungs of mice exposed to the high concentration of oxygen remained unchanged compared with levels measured in animals breathing room air. In striking contrast, however, levels of IsoFs in the lungs increased significantly over control levels after just 3 h of oxygen exposure (Fig. 6). Subsequent experiments have identified important subcellu-

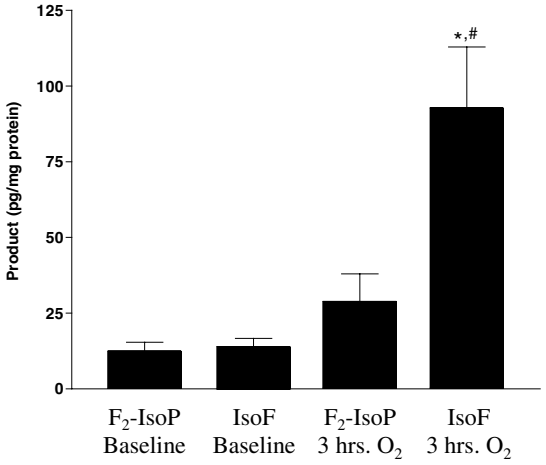

FIG. 6. IsoFs and F_2-IsoPs esterified in lung following oxygen exposure. C57/Bl6 mice ($n = 8$ in each group) were analyzed at baseline and after 3 h of exposure to >98% oxygen. F_2-IsoPs show a modest, but statistically insignificant, trend toward increase, whereas IsoFs increase dramatically after just 3 h of oxygen exposure. *$p < 0.001$ versus IsoF baseline; #$p < 0.01$ versus F_2-IsoP 3 h of O_2.

lar targets of oxidative injury and have shown that the pulmonary endothelium is an especially important site of damage following brief exposure to high concentrations of oxygen (unpublished observations).

These studies taken together have several potentially important clinical implications. First, these findings suggest that oxygen begins causing damage to the lung that can be detected by enhanced formation of IsoFs, but not F_2-IsoPs, long before symptoms of lung injury become clinically apparent. Furthermore, damage is detectable after exposure to a high concentration of oxygen for only a short period that is generally considered safe by clinicians. Mention should be made that these results were obtained in mice, and it remains to be shown whether these findings are also applicable to humans. If, however, even relatively brief exposures to high concentrations of oxygen have the potential to cause damage at the cellular level in humans, as in mice, which can be both dose- and time-dependent, perhaps oxygen should be considered as a "drug" that should be used as cautiously as any other more traditional pharmacological intervention. Finally, these experiments establish the occurrence of oxidant injury in the setting of hyperoxia and suggest that quantification of IsoFs provides a remarkably sensitive indicator of hyperoxia-induced oxidant injury. This also opens up the possibility of utilizing measurements of IsoFs to explore therapeutic interventions that may minimize pulmonary oxygen toxicity, allowing the safe delivery of higher concentrations of oxygen in critically ill patients.

SUMMARY AND FUTURE INVESTIGATIONS

Our investigations to date have characterized IsoFs as novel products of free radical-catalyzed peroxidation of arachidonic acid *in vitro* and *in vivo*. IsoFs have been characterized structurally and have been shown to contain a substituted tetrahydrofuran ring. The IsoFs have been shown to exhibit favored formation with increasing oxygen tension, whereas the formation of F_2-IsoPs becomes disfavored in the presence of elevated concentrations of oxygen. The ability of ambient oxygen concentration to modulate the formation of both IsoPs and IsoFs has also been demonstrated *in vivo*, and the IsoF/F_2-IsoP ratio can reflect steady-state tissue oxygenation. Quantification of IsoFs has strongly implicated a role for oxidant injury in the pathogenesis of two distinctly different disorders, PD and hyperoxia-induced lung injury, and these observations would not have been possible using only measurements of F_2-IsoPs. Moreover, these initial lines of inquiry have opened up a number of additional avenues for future investigation, some of which are discussed below.

Many additional opportunities for investigation that have evolved from the discovery of IsoFs remain to be explored. One of the most interesting and important questions remaining to be addressed is whether IsoFs are not just biomarkers of lipid peroxidation, but whether they also exert biological effects and thus might participate as mediators of oxidant injury, as has been found for IsoPs. This question has remained thus far unanswered due to the lack of a synthesized pure IsoF isomer. During *in vitro* oxidation of arachidonic acid, 128 chemically distinct isomers of IsoFs are formed, in addition to a myriad of other products of lipid peroxidation. Therefore, any attempt to purify a single IsoF isomer to sufficient purity and in sufficient quantities to perform bioassays would be prohibitively difficult, if not impossible. As mentioned above, however, a single IsoF isomer has been synthesized recently that will now allow us to begin to explore whether it is capable of exerting biological actions.

The studies of IsoFs in pathologic states conducted to date have provided insights that have engendered a number of additional hypotheses and avenues for further investigation. These include genetic and drug-induced mitochondriopathies and the opportunity to define the relationship between concentration of oxygen and time of exposure in regard to risk for oxidative injury to the lung in humans. What has clearly emerged from the discovery of IsoFs and studies performed thus far is that measurement of F_2-IsoPs alone as an indicator of oxidative stress has its limitations as does measurement of IsoFs, but that combined measurement of both F_2-IsoPs and IsoFs likely provides the most accurate and reliable quantitative index of oxidative stress status available to us at the present time.

ABBREVIATIONS

AD, Alzheimer's disease; DLB, diffuse Lewy body disease/dementia with Lewy bodies; GC/NICI/MS, gas chromatography negative ion chemical ionization mass spectrometry; IsoF, isofuran; IsoP, isoprostane; MSA, multiple system atrophy; PD, Parkinson's disease; SN, substantia nigra.

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