Modification of Proteins by Isoketal-containing Oxidized Phospholipids*

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Oxidative stress frequently leads to altered function of membrane proteins. Isoketals are highly reactive products of the isoprostane pathway of free radical-induced lipid peroxidation that rapidly form covalent protein adducts and exhibit a remarkable proclivity to form protein cross links in vitro. Examination of isoketal adducts from an animal model of oxidative injury revealed that initial adducts were formed by isoketals esterified in phospholipids, representing a novel oxidative injury-associated modification of proteins by phospholipids. Maturation of adducts involved cleavage from phospholipids and conversion of adducts to a more stable chemical form that can be detected for extended periods. Because initial adducts were formed by phospholipid-esterified isoketals, the functional consequence of isoketal adduction was examined using a model membrane protein (a cardiac K+ channel). These studies revealed that isoketal adduction profoundly altered protein function, inhibiting potassium current in a dose-dependent manner. These findings indicate that phospholipid-esterified isoketals rapidly adduct membrane proteins and that such modification can alter protein function, suggesting a generalized cellular mechanism for alteration of membrane function as a consequence of oxidative stress.

Oxidative stress from the generation of free radicals has been implicated in the pathogenesis of a wide variety of human diseases including atherosclerosis, cancer, and neurodegenerative diseases (1–3). Fatty acids esterified in membrane phospholipids are a major target of attack by free radicals (4–6). We have described previously the formation of a series of highly electrophilic γ-ketoaldehyde isomers in vitro, which we term isoketals (IsoKs) (7), by rearrangement of endoperoxide intermediates in the isoprostane pathway of free radical-induced peroxidation of phospholipid-esterified arachidonic acid (8). Incorporation of oxygen at different sites of arachidonyl radical formation results in the formation of 8 structural isomers, each of which is composed of 4 racemic diastereoisomers (see Fig. 1A). Analogous molecules, termed neuroketals, are formed from the free radical-catalyzed oxidation of docosahexaenoic acid and form protein adducts analogous to those described below for IsoKs (9).

IsoKs adduct covalently with the e-amino group of lysine residues in vitro within seconds, which is orders of magnitude faster than has been reported for other products of lipid peroxidation, including 4-hydroxynonenal (7). This remarkable reactivity has precluded the detection of free IsoKs in vivo because of their sequestration as adducts. Therefore, we elucidated the nature of IsoK protein adducts in vitro as an initial step toward identifying the formation of IsoK adducts in vivo (7, 10). IsoKs initially form reversible Schiff base adducts which irreversibly cyclize to pyrroles. The pyrroles then undergo facile autoxidation to stable lactam and hydroxylactam adducts (see Fig. 1B) (7, 10). Consistent with this process, Schiff base adducts form rapidly and then undergo a rapid decline that is accompanied by an accumulation of lactam adducts (10). Accordingly, Schiff base adduct levels provide an indicator of early immediate adduction, whereas levels of lactam adducts provide an index of more chronic ongoing adduct formation. Here we report that in an animal model of oxidative injury, phospholipid-esterified IsoKs rapidly form Schiff base adducts, presumably with adjacent membrane proteins. The initial adducts are cleaved from the phospholipid backbone, resulting in IsoK adducts that are found to accumulate in plasma from normal humans and rats. Furthermore, IsoK ad- duction is found to alter the function of a model membrane protein, a cardiac K+ channel.

EXPERIMENTAL PROCEDURES

Preparation and Analysis of Rat Liver Proteins—Male Sprague-Dawley rats were fasted overnight and then given carbon tetrachloride (1 ml/kg diluted 1:1 with corn oil) intragastrically. Four h later, rats were anesthetized with pentobarbital (65 mg/ml) and then exsanguinated. Livers were removed and frozen in liquid nitrogen. Liver (1–2 g) was ground in ice-cold Folch solution (20 ml/g) containing 5 mg of butylated hydroxytoluene and 50 mg of triphenylphosphine/100 ml and incubated for 30 min at room temperature under argon. Proteins were then collected by vacuum filtration and washed with 100 ml each of Folch solution and ethanol (each containing butylated hydroxytoluene and triphenylphosphine). Proteins were suspended in 3 ml of methanol (5 mg of butylated hydroxytoluene/100 ml) and 3 ml of 0.4 M sodium hydroxide and hydrolyzed at room temperature under argon for 2 h. In samples in which Schiff base adducts were measured, proteins were reduced with 0.1 volume sodium borohydride (1N in dimethylformamide for 30 min) before base treatment. Proteins were then reprecipitated and washed as above before resuspension at 2 mg/ml in phosphate-buffered saline, pH 7.4. Labeled ([15N2,13C6] IsoK lysyl lactam (14.48 ng) and reduced anhydro Schiff base adduct standards (47.80 ng) (synthesis, see below) were added, and the proteins were heated to 95 °C for 10 min. After cooling, Pronase (Calbiochem) was added (0.1 volume at 1 mg/ml), and the mixture was incubated overnight at 37 °C under argon. The digest was then heated to 95 °C for 10 min. After cooling, aminopeptidase M (1 mg/ml) was added, and the mixture was incubated overnight at 37 °C under argon. The digest was applied to
Oasis cartridges (Waters Associates). Adducts were eluted with 10 ml of methanol and dried under N2. Adducts were purified by isocratic HPLC (45% methanol in 20 mM ammonium acetate with 0.1% acetic acid) on a 4.6 × 250-mm Macrosphere 300 C18 column (Alltech Association). One-ml fractions were collected, and fractions containing radioactivity were combined, re-extracted with Oasis cartridges, and analyzed by liquid chromatography tandem mass spectrometry as previously reported (7, 10).

Electrophysiology—Electrophysiological recordings were performed at room temperature using an Axopatch-200A patch-clamp amplifier (Axon Instruments, Inc.) in the whole cell configuration of the patch-clamp technique. To record $I_{kr}$, Tyrode’s solution was used as the extracellular solution. The intracellular pipette filling solution contained 145 mM K.

Results and Discussion

We addressed whether highly reactive IsoKs are formed in vivo in rats treated with CCl₄, a well established model of oxidant injury to the liver that is associated with marked overproduction of isoprostanes (8, 12). Analysis of IsoK protein adducts involves quantification of IsoK-lysyl adducts following enzymatic digestion of proteins to individual amino acids. A unique aspect of isoprostanes and, thus also, IsoK formation is that they are initially formed in situ esterified to phospholipids followed by phospholipase release (13). Because of their high

Preparation and Analysis of Serum Proteins—Plasma from normal rats and humans was delipidated in Folch solution containing butylated hydroxytoluene and triphenylphosphine at −20 °C (1 ml of plasma/20 ml). Proteins were precipitated and further treated as above.

Electrophysiology—Electrophysiological recordings were performed at room temperature using an Axopatch-200A patch-clamp amplifier (Axon Instruments, Inc.) in the whole cell configuration of the patch-clamp technique. To record $I_{kr}$, Tyrode’s solution was used as the extracellular solution. The intracellular pipette filling solution contained 145 mM K. Pulses of a 1-s duration from a holding potential of −40 mV were used. Tail currents were measured as the difference between current recorded immediately after a −40-mV step and holding current.

RESULTS AND DISCUSSION

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reactivity, we considered that IsoKs might rapidly adduct to membrane proteins while still esterified to phospholipids, forming a heterotrimeric complex consisting of phospholipid-IsoK-protein. If this occurs, adducts would not be detected unless the complex is hydrolyzed to free the protein adduct from the phospholipid. Hence, IsoK protein adducts were analyzed in liver with and without base hydrolysis. Lysyl adducts of proteins were isolated, purified, and then quantified by liquid chromatography tandem mass spectrometry using selected reaction monitoring of fragmentation of the MH$^+$ ion to specific daughter ions for the lactam and reduced Schiff base adducts as described previously (7, 10).

Representative selected reaction monitoring ion current chromatograms obtained from the analysis of IsoK lactam and Schiff base adducts in liver are shown in Fig. 2, A and B, respectively. The chromatogram in Fig. 2A was obtained from analysis of liver from a control untreated rat, and the chromatogram in Fig. 2B was from the liver of a CCl$_4$-treated rat. Schiff base adducts were not detected in normal rat liver (Fig. 2D). However, lactam adducts were detected at a level of 3.5 ± 0.2 ng/g of tissue (mean ± S.E.; n = 4), and none were esterified to phospholipids (Fig. 2C). This indicated that IsoK lactam adducts accumulate in the normal state because of ongoing lipid peroxidation, as has been found for isoprostanes (8, 12, 13).

A very different picture emerged in rats treated with CCl$_4$. Four h following administration of CCl$_4$, lactam adduct levels in the liver were 6.4 ± 0.3 ng/g (n = 4), representing an increase of 82% above the levels in normal livers (p = 0.0003). Again, phospholipid-esterified adducts were not observed (Fig. 2C). Although Schiff base adducts were undetectable in normal liver, abundant quantities were present in livers from rats treated with CCl$_4$. In striking contrast to lactam adducts, >97% of Schiff base adducts were esterified to phospholipids. In the absence of treatment of proteins with base to release phospholipids, the level detected was 0.5 ± 0.1 ng/g of tissue (n = 4). When proteins were treated with base, the level detected increased dramatically by a mean of 42-fold to 21 ± 4 ng/g.
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ng/g of tissue (n = 8) (Fig. 2D). This finding suggests that IsoKs esterified in phospholipids rapidly adduct as Schiff bases to adjacent proteins, presumably membrane-associated, and then convert to lactams and are hydrolyzed from the phospholipid backbone. This is consistent with our finding that phospholipid-esterified lactam adducts are substrates for snake and bee venom phospholipase A₂ enzymes in vitro.²

Because IsoK lactam protein adducts were detected in normal liver and were not sequestered as a complex with phospholipids, we hypothesized that they may also accumulate in the circulation. Therefore, we analyzed normal plasma for IsoK lactam adducts and found levels of 199 ± 78 pg/ml (n = 4) in rat plasma and levels of 561 ± 101 pg/ml (n = 5) in human plasma. This provides evidence that IsoK adducts are formed chronically in physiologically unperturbed rodents and humans.

Because membrane-associated proteins are presumably the preferential target for adduction by IsoKs, we tested the functional consequence of adduction using a model integral membrane potassium channel that is relevant to oxidative ischemia/reperfusion injury-induced cardiac dysrhythmias (14, 15). We tested the effect of a synthetic IsoK (box-enclosed compound Fig. 1A) on the rapidly activating delayed rectifier K⁺ current (I₅⁰) expressed in AT-1 cells, an atrial tumor myocyte cell line (14, 15). Whole cell patch-clamp experiments following addition of the IsoK revealed a pronounced dose-dependent inhibition of I₅⁰ (IC₅₀ = 2.2 μM) (Fig. 3, A and C). This occurred despite the fact that addition of free IsoK does not mimic the formation of Schiff base protein adducts esterified to membrane phospholipids, which would be expected to have more disruptive effects on protein structure and function. Activating and deactivating currents were suppressed equally, suggesting that these effects were neither voltage- nor gating-dependent. Moreover, I₅⁰ was not restored with washing, suggesting that the inhibition was not caused by a reversible allostERIC effect but rather an irreversible covalent modification of channel proteins (Fig. 3B). The inhibition was not manifested immediately, requiring 60 min to achieve full inhibition when tested at a concentration of 10 μM IsoK. Because IsoKs adduct to proteins within seconds, the more prolonged time course for I₅⁰ inhibition may be caused by time-dependent diffusion into the membrane and/or cross-linking of channel proteins. The latter possibility would be consistent with the observation that these compounds exhibit a remarkable proclivity to cross-link proteins after the initial adduct is formed (7).

Oxidative stress has been found to induce alterations in the function of a number of membrane proteins including ion channels, enzymes, and receptors (16–20). Electrophile short chain aldehydes produced by lipid peroxidation have been shown to be capable of producing some of these effects when added to cells (21–26). However, IsoKs are more likely to mediate these effects for a number of reasons. First, the short chain aldehydes are scission products of oxidized fatty acids in phospholipids and thus are released from the fatty acid backbone as they are formed (27). Furthermore, these short chain aldehydes are orders of magnitude less reactive and are more hydrophilic than IsoKs, which would permit their diffusion from the membrane before adducting to proteins. Consistent with this hypothesis, these short chain aldehydes, unlike IsoKs, can be detected as free unadducted compounds in biological fluids and tissues (27).

In summary, these findings provide evidence that IsoKs are formed in vivo and rapidly adduct to membrane proteins while still esterified to phospholipids. This novel protein modification by phospholipids provides new insights into potential underlying mechanisms by which oxidative stress can induce structural and functional modification of membrane proteins, ultimately causing cellular dysfunction.

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