Chapter 2
Modulation of Protein Function by Isoketals and Levuglandins

Sean S. Davies

Abstract Oxidative stress, defined as an increase in reactive oxygen species, leads to peroxidation of polyunsaturated fatty acids and generates a vast number of biologically active molecules, many of which might contribute in some way to health and disease. This chapter will focus on one specific class of peroxidation products, the levuglandins and isoketals (also called isolevuglandins). These γ-ketoaldehydes are some of the most reactive products derived from the peroxidation of lipids and exert their biological effects by rapidly adducting to primary amines such as the lysyl residues of proteins. The mechanism of their formation and remarkable reactivity will be described, along with evidence for their increased formation in disease conditions linked with oxidative stress and inflammation. Finally, the currently known effects of these γ-ketoaldehydes on cellular function will then be discussed and when appropriate compared to the effects of α,β-unsaturated fatty aldehydes, in order to illustrate the significant differences between these two classes of peroxidation products that modify proteins.

Keywords Aldehydes · isoketals · levuglandins · lipid peroxidation · protein modification

2.1 Introduction

2.1.1 Mechanisms of Isoketal and Levuglandin Formation

Conversion of arachidonic acid to an eicosanoid γ-ketoaldehyde can proceed by two separate pathways, one driven enzymatically by cyclooxygenases and the other driven non-enzymatically by free radicals. The two pathways differ only in the mechanism used to generate the key intermediate, a bicyclic endoperoxide with two aliphatic side chains, which can undergo non-enzymatic rearrangement to

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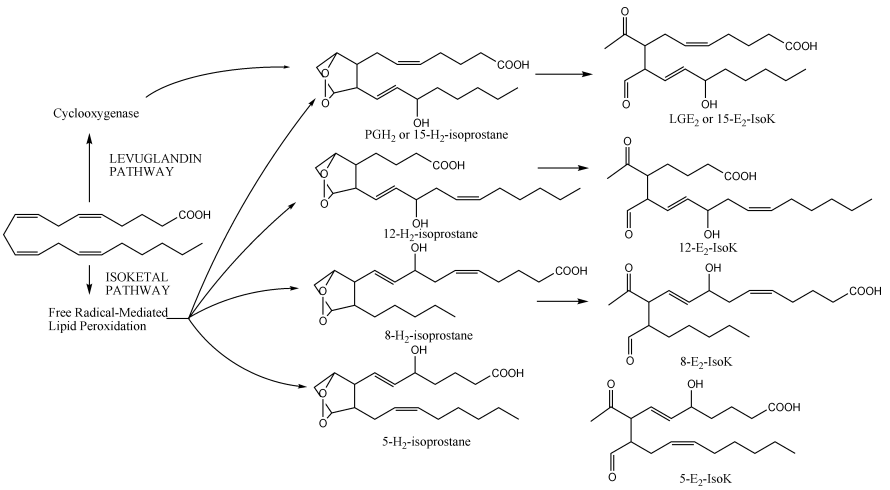


Fig. 2.1 Formation of levuglandins (LG) and isoketals (IsoK) from arachidonic acid. Arachidonic acid can be converted to a γ-ketoaldehyde by two distinct pathways. In the levuglandin pathway, cyclooxygenase enzymes convert arachidonic acid to PGH_2, which then rearranges non-enzymatically to form LGs. In the isoketal pathway, free radicals mediated lipid peroxidation forms four different regioisomers of H_2-isoprostanes, that also rearrange non-enzymatically to form IsoKs. The different regioisomers are identified by the position of their hydroxyl group. 15-H_2-isoprostane is the same regioisomer as PGH_2, so that one of the eight stereoisomers of 15-E_2-IsoK is identical to LGE_2

form the γ-ketoaldehyde. In the enzymatic pathway, cyclooxgenases convert non-esterified arachidonic acid to the bicyclic endoperoxide, prostaglandin H_2 (PGH_2) (Fig. 2.1). In the non-enzymatic pathway, free radical mediated peroxidation of esterified and non-esterified arachidonic acid generates four PGH_2-like regioisomers (H_2-isoprostanes) (Morrow et al., 1990). The specific regioisomer formed depends on which of the bis-allylic hydrogen the free radical initially attacks and the different H_2-isoprostane regioisomers are denoted by the carbon number of their hydroxyl group. These bicyclic endoperoxides have 4 stereocenters, so that each radical generated regioisomer includes 16 stereoisomers for a total of 64 H_2-isoprostane isomers compared to the single stereoisomer, PGH_2, generated by the cyclooxygenases.

After formation of the bicyclic endoperoxides, formation of the γ-ketoaldehydes proceeds by an identical non-enzymatic concerted scission rearrangement for both pathways (Fig. 2.2) (Salomon et al., 1984). Because base-catalyzed rearrangement can be initiated on either side of the nearly symmetrical bridgehead, both E_2- or D_2- isomers of γ-ketoaldehyde form. E_2-isomers have the ketone group adjacent to the carboxylate side chain and the aldehyde adjacent to the second side chain, while D_2-isomers have the aldehyde group adjacent to the carboxylate side chain and the ketone group adjacent to the second side chain. In the case of PGH_2 rearrangement, the two isomers

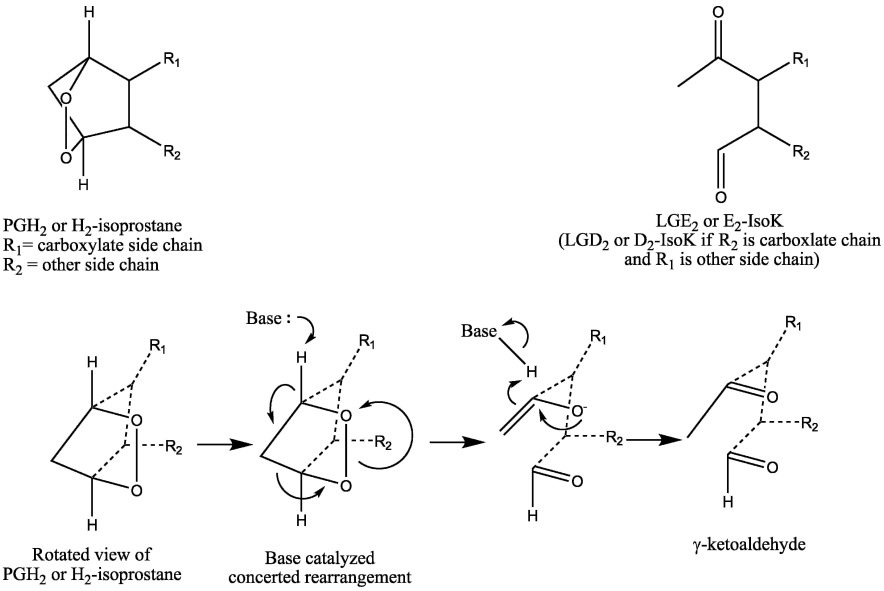


Fig. 2.2 Rearrangement of bicyclic endoperoxides to form γ-ketoaldehyde. Proposed mechanism for formation of γ-ketoaldehydes from bicyclic endoperoxide such as PGH_2 and H_2-isoprostanes by base-catalyzed concerted rearrangement (Salomon et al., 1984). Attack of base is shown at the top of the bridgehead, which results in formation of E_2-isomers. Attack at the symmetrical position below the bridgehead forms the D_2-isomers

are called levuglandin (LG) E_2 and D_2. With H_2-isoprostane rearrangement, the resulting collective set of 64 regio- and stereo-isomers are given the trivial name E_2- and D_2-isoketals (IsoK) or alternatively isolevuglandins (Brame et al., 1999). As with H_2-isoprostanes, D_2- and E_2-IsoK regioisomers are denoted by the position of the hydroxyl group, so that 15-E_2-IsoK represents the same regioisomer as LGE_2, and in fact, one of the eight stereoisomers of 15-E_2-IsoK is structurally identical to LGE_2. Finally, it should be noted that peroxidation of docosahexanoic acid (DHA) generates similar γ-ketoaldehydes designated as neuroketals (Bernoud-Hubac et al., 2001).

Incubation of PGH_2 in phosphate buffer yields 22% LG (Salomon et al., 1984). Incubation of PGH_2 in DMSO, which may better model the hydrophobic environment near cyclooxygenases, yields 70% LG. The non-enzymatic rearrangement of PGH_2 to LG occurs even in cells with active prostaglandin synthases. For instance, stimulated platelet produce prodigious amounts of thromboxane through the cyclooxgenase/thromboxane synthase pathway, yet platelet stimulation also produces significant amounts of LG (Boutaud et al., 2003). LG formation can be enhanced in this system by inhibition of thromboxane synthase. Because prostaglandin synthases do not act on H_2-isoprostanes, the relative yield of IsoK derived from H_2-isoprostane formed in cells is expected to be similar to that found in vitro.

In summary, γ-ketoaldehydes can be formed by two pathways whose products can only be distinguished by the number of isomers formed and by the potential presence of esterified forms for IsoKs. So far, no experimental data has shown that the biological effects of the γ-ketoaldehydes are specific only to a particular regio- or stereo-isomers of the γ-ketoaldehydes, so that the blanket term IsoK/LG will be used in this review even when a specific effect was demonstrated using a particular LG or IsoK species.

2.1.2 Cardinal Features of IsoK/LG Reaction with Proteins

Interest in the biological activity of IsoK/LGs stems from the cardinal features of IsoK/LG biochemistry: (1) their extremely rapid adduction to proteins, (2) their proclivity to crosslink proteins, and (3) their propensity to disrupt protein function.

2.1.2.1 IsoK/LG Rapidly Adduct to Proteins

IsoK/LGs react nearly instantaneously with primary amines such as the lysyl residues of proteins. Although 4-hydroxynonenal (HNE), an α,β-unsaturated aldehyde that also forms by lipid peroxidation, is often considered to be highly reactive, it reacts at a very pedestrian rate when compared to IsoK/LG. For instance, when IsoK/LG or HNE is added to human serum albumin, the half-life of unadducted IsoK/LG is less than 20 s, while the half-life of HNE is about 60 min (Brame et al., 1999). In practical terms, the difference in reactivity between IsoK/LG and HNE means that only adducted IsoK/LG can be found in vivo, while unadducted HNE can be readily measured in tissues and plasma. Additionally, the reactivity of IsoK/LG precludes significant diffusion, so that only the proteins nearest the sites of IsoK/LG formation, such as membrane-associated proteins, are likely to be adducted.

The rapid reaction rate of IsoK/LG is driven by the stability of the pyrrole adducts formed (Fig. 2.3). In common with all aldehydes including HNE, an initial nucleophilic attack by the lysyl nitrogen forms a hemiaminal adduct which dehydrates to an imine (Schiff base) adduct. This highly reversible reaction product can usually only be measured after conversion to a reduced Schiff base by a strong reducing agent such as sodium borohydride. What makes IsoK/LGs so much more reactive than ordinary aldehydes? With γ-ketoaldehydes, formation of the initial hemiaminal adduct positions the second carbonyl group to also undergo nucleophilic attack. This pyrrolidine adduct then quickly undergoes dehydration to form a pyrrole, making the reaction essentially irreversible. For other aldehydes such as HNE, no secondary nucleophilic attack is possible, so the unstable hemiaminal adduct

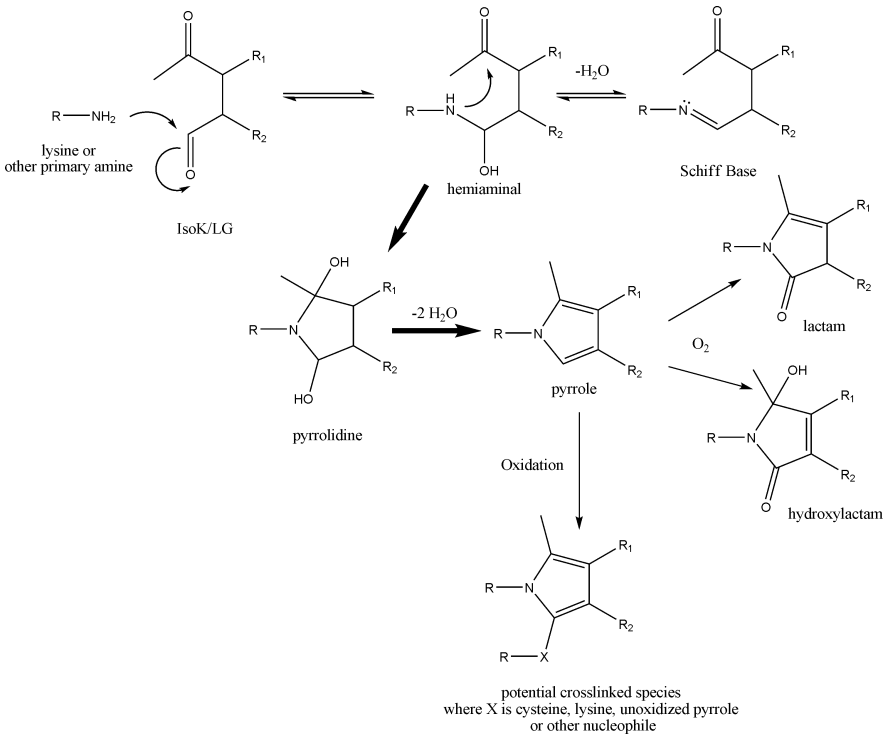


Fig. 2.3 Reaction of IsoK/LG with primary amines to form stable adducts. Primary amines including lysine react with IsoK/LGs to form a hemiaminal adduct. Unlike most aldehydes which can only form the highly reversible Schiff base adduct, the hemiaminal adduct of γ-ketoaldehydes can undergo a second nucleophilic attack to form a pyrrolidine adduct which dehydrates to form an irreversible pyrrole adduct. In the presence of oxygen, the pyrrole is converted to lactam and hydroxylactam adducts. Oxidation of the pyrrole leads to formation of stable crosslinked species

simply reverses back to unadducted HNE and lysine. Stable adduction of protein by HNE and related α,β-unsaturated aldehydes generally occurs through a Michael addition reaction, for which thiols are more reactive nucleophiles than lysines.

The rate of pyrrole adduct formation can be determined by addition of the Ehrlich reagent that reacts with pyrroles to form a visibly purple product (DiFranco et al., 1995). In the presence of oxygen, the ability of adduct to react with the Ehrlich reagent diminishes over time. Analysis by mass spectrometry found that in the presence of oxygen, the pyrrole adduct goes on to form highly stable lactam and hydroxylactam adducts (Fig. 2.3) (Brame et al., 1999). Therefore, quantification of lactam adducts, rather than pyrrole adducts, is probably most useful except in artificial conditions when oxygen can be completely excluded.

2.1.2.2 IsoK/LG Crosslink Proteins

Another cardinal feature of IsoK/LG biochemistry is the proclivity to form crosslinked protein aggregrates. This feature can be readily appreciated by dose curves from treatment of a model protein such as chicken egg ovalbumin with increasing molar equivalents of IsoK/LG (Davies et al., 2002; Iyer et al., 1989). At low molar equivalents of IsoK/LG, there is a small proportion of protein that migrates on SDS-PAGE with the apparent mass of dimers and trimers that indicate intermolecular crosslinking. At ten or greater molar equivalents, most of the protein runs as highly oligimerized forms of the protein. Because each molecule of ovalbumin is estimated to have twenty surface lysines, saturation of available surface lysines is not required to generate extensive crosslinking.

The mechanism that underlie this proclivity to crosslink is believed to be oxidation of the pyrrole adduct to form electrophiles that can readily react with nucleophiles including thiols, amines, or unoxidized pyrroles (Fig. 2.3) (Amarnath et al., 1994). Therefore, IsoK/LG can readily crosslink proteins not only to adjacent proteins but to DNA or polyamines as well (Boutaud et al., 2001; Murthi et al., 1993). Detection of IsoK/LG intermolecular crosslinks currently relies on visualization of co-migrating species on SDS-PAGE. Unfortunately, the exact molecular species of the crosslinks has eluded characterization by mass spectrometry or NMR. One reason for this failure is the difficulty of working with highly crosslinked material. The purple-to-brownish crosslinked material typically forms insoluble aggregates that do not pass through solid phase extraction cartridges and HPLC columns and that poorly ionize in mass spectrometers.

The conditions that facilitate the formation of lactams versus crosslinks are poorly characterized but are likely to be mediated by the proximity of lysine and other nucleophiles. Increasing the molar equivalents of IsoK/LG versus lysine residues significantly increases the extent of intermolecular crosslinking visualized by SDS-PAGE, but also linearly increases the amount of lactam adduct measured (Davies et al., 2007). Thus measurement of IsoK-lysyl-lactam appears to be a reasonable surrogate marker for all other IsoK/LG adducts formed, and increases in IsoK-lysyl-lactam in tissues or cells most likely also indicates increases in IsoK/LG crosslinked proteins as well.

2.1.2.3 Disruption of Protein Function

A final cardinal feature of IsoK/LG biochemistry is their propensity to disrupt protein function. Modification of proteins by IsoK/LG could theoretically alter protein function by several mechanisms. Adduction of IsoK/LG to lysine converts a short, positively charged group to a bulky, hydrophobic, negatively charged group. Modifications of lysyl residues in the active site of enzymes will therefore eliminate catalytic activity. Catalytic activity may be lost even if the modified residue is simply adjacent to the active site, as the bulky IsoK/LG adduct may sterically hinder substrate binding or product release. Protein

modification by IsoK/LG may also disrupt protein function by altering protein conformation. Crosslinking of nearby cysteine and lysyl residues could significantly deform the conformation and lock the protein in an active or inactive conformation. Intermolecular crosslinking that initiates protein aggregates may not only alter conformation, but potentially initiate cellular stress responses.

In addition to directly altering catalytic activity, IsoK/LG modification may alter protein function by changing interactions with other regulatory proteins. For example, modification of lysines required for protein-protein interactions will disrupt this interaction. Depending on the specific protein, such disruption could be inhibiting or activating. Similarly, addition of a bulky hydrophobic group could also cause the adducted protein to more strongly partition to membranes, thus altering interaction with normal binding partners and creating novel partners. Finally, adduction of IsoK/LG to lysyl groups may alter the degradation rate or pathways of adducted proteins. In doing so, it may significantly prolong or shorten the half-life of the adducted protein and thereby lead to dysregulation.

2.1.3 IsoK/LG Protein Adducts form in Various Disease Conditions

Because of the myriad ways that IsoK/LG adduction might disrupt normal physiological proteins, a rational exploration of how IsoK/LGs contribute in disease processes first requires defining conditions where IsoK/LG adducts are increased. Currently, there are two complimentary methods for quantifying IsoK/LG adducts in vivo. The first method utilizes the sensitivity and specificity of electrospray ionization tandem mass spectrometry. Quantitative measurement of IsoK-lysyl-lactam adducts can be made by complete enzymatic proteolysis of tissue followed by partial purification on solid phase extraction cartridges and HPLC and then analysis by mass spectrometry (Davies et al., 2007). A heavy isotope labeled internal standard is added to the sample for quantification. The second method utilizes antibody based approaches such as ELISA, Western blotting, or immunohistochemistry. Anti-IsoK/LG antibodies are made by immunizing animals with IsoK/LG adducted proteins or by screening single-chain antibody libraries with IsoK/LG adducted peptides.

Both quantitative approaches have drawbacks. For instance, the current mass spectrometric method does not distinguish between the various isomers of IsoK/LG adducts and thus provides no information on the pathway leading to adducts. Because complete proteolyis is required, it is also not possible to specifically determine the identity of the adducted protein. The method is also very time-consuming and limited to labs with electrospray ionization tandem mass spectrometers and associated expertise. In contrast to mass spectrometry methods, antibody based methods are relatively easy and inexpensive; however,

in comparison to anti-protein antibodies, anti-lipid antibodies tend to suffer from relatively poor affinity and greater non-specificity. The poor performance of anti-lipid antibodies is likely inherent to the chemical properties of the antigen, as lipid antigens lack the high number of rigid structures that are typically required for high affinity, high specificity antigen binding. The only rigid structure in the IsoK/LG adducts are the pyrrole or lactam ring, structures shared with other biologically relevant compounds such as porphyrins. Nevertheless, the presence of aliphatic side chains in IsoK/LG adducts appear to confer reasonable specificity as antibodies with selectivity for separate regioisomers of IsoK/LG adducts have been reported (DiFranco et al., 1995; Poliakov et al., 2004; Salomon et al., 1997b; Salomon et al., 1999; Salomon et al., 2000). Ideally, both mass spectrometric and antibody methods would be used to confirm increases in specific disease conditions.

IsoK/LG adducts increase in a number of conditions related to oxidative stress and inflammation. The first published report of IsoK/LG adducted proteins in vivo was in very small groups of patients with documented atherosclerosis or end-stage renal disease using ELISA measurements (Salomon et al., 1997b). LGE_2 adducted keyhole limpet hemocyanin was the immunizing antigen for the antibody utilized in this study, therefore both the IsoK and LG pathways might have contributed to detected immunoreactivity. A follow-up study also used a second antibody that recognized an IsoK regioisomer adduct that could only be derived from the IsoK pathway. In this study, both of the antibodies measured an approximately two-fold change in plasma adduct levels in the atherosclerotic and renal disease patients compared to controls (Salomon et al., 2000). This finding implicates the IsoK pathway, rather than the LG pathway, as the major source of adducts in these diseases.

The first study demonstrating IsoK/LG adducts in vivo using mass spectrometric methods used carbon chloride treated rats (Brame et al., 2004). Carbon tetrachloride (CCl_4) is converted to trichloromethyl free radical by cyctochrome P450s in the liver, leading to a massive increase in lipid peroxidation. After four hours of treatment with either CCl_4 or vehicle, the rats were sacrificed and a portion of the recovered livers reacted with sodium borohydride to allow measurement of reduced Schiff Base adducts. Additionally, a portion of liver protein extracts were base hydrolyzed in order to measure total (both esterified and non-esterified) IsoK adducts. In livers from untreated animals, no reduced Schiff base adducts was detected, but lactam adduct was measured to be 3.5±0.2 ng/g tissue. In CCl_4 treated animals, the total reduced Schiff base adduct was 21±4 ng/g tissue, with only 0.5±0.1 ng/g tissue being non-esterified. Non-esterified lactam adduct was 6.4±0.3 ng/g and similar levels of total lactam adduct were found. This result is consistent with the notion that immediately following CCl_4 treatment, IsoKs initially form in situ on phospholipids and are still esterified when they react with proteins to form Schiff base adducts. The presence of lactam adduct even in untreated animals suggests that the lactam adduct is stable and can thus accumulate during basal rates of lipid peroxidation in vivo. That only non-esterified

lactam adduct is found even after CCl_4 treatment suggests the presence of a phospholipase that can act on either the pyrrole or lactam adduct to hydrolyze the modified phospholipid. While no further studies have been carried out to confirm the existence of this phospholipase, an attractive candidate would be platelet-activating factor acetylhydrolase, which hydrolyzes other phospholipid-esterified isoprostane products.

Increased tissue levels of IsoK/LG protein adducts have been found in a number of other conditions associated with oxidative stress and inflammation including ischemic heart (Fukuda et al., 2005), hyperoxic lung (Davies et al., 2004), Alzheimer's Disease brain (Zagol-Ikapitte et al., 2005), experimental sepsis plasma (Poliakov et al., 2003), allergic inflammation lung (Talati et al., 2006), and glaucomatous trabecular meshwork (Govindarajan et al., 2008). From this limited sampling, it seems reasonable to expect increases in other conditions where oxidative stress or inflammation occur. While the demonstration of increased IsoK/LG is consistent with their contribution to the disease process, these results by themselves do not provide conclusive evidence as to whether IsoK/LG adducts have any real pathophysiological significance or are simply tombstones of more important events. This important question must be addressed in two ways: first, by demonstrating in cellular assays plausible mechanisms for IsoK/LG adduction to contribute to pathophysiology and second, by demonstrating that reducing IsoK/LG adducts, preferably without altering other oxidative events, significantly ameliorates pathophysiology.

2.1.4 Other Primary Amines Compete with Lysines for Adduction to IsoK/LGs

2.1.4.1 Other Endogenous Amines React with IsoK/LGs

Although the lysyl residues of proteins are probably the most important target of IsoK/LG in the cell, IsoK/LG reacts with a wide range of primary amines. Another abundant primary amine in cells is the ethanolamine head group of phosphatidylethanolamine (PE). Because IsoKs form on membrane phospholipids, they would be well-positioned to react with PE. Incubation of one molar equivalent of IsoK/LG and 1-palmitoyl, 2-linoleolyl-PE for two hours produced a stable IsoK/LG-PE pyrrole adduct (Bernoud-Hubac et al., 2004). No evidence of a lactam or hydroxylactam adduct was found under these conditions, likely because the reaction conditions were designed to exclude oxygen. The formation of IsoK/LG-PE is of interest because oxidatively modified phospholipids have been implicated in certain autoimmune diseases such as lupus. Whether this reaction actually occurs in vivo has yet to be determined.

IsoK/LG can also react with arginine to form a bis-IsoK/LG-arginine adduct which then undergoes decomposition to a bis-IsoK/LG-urea (BLU)

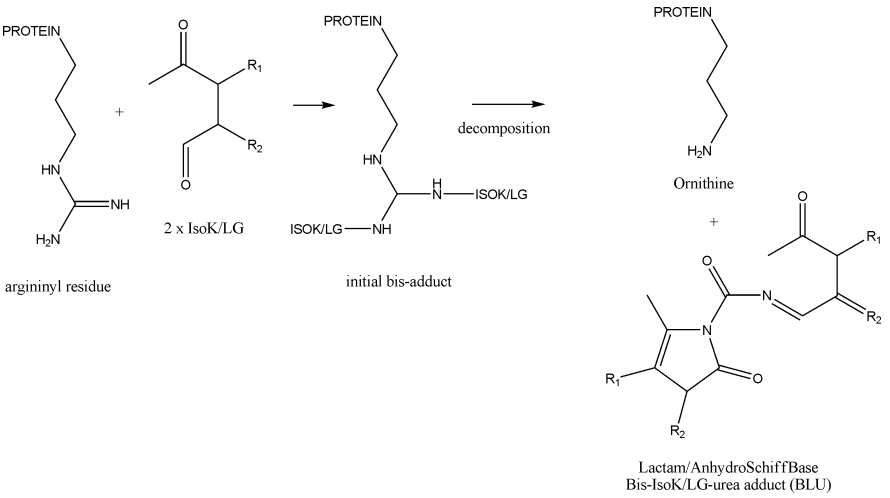


Fig. 2.4 Reaction of IsoK/LG with arginine. According to proposed mechanism (Zagol-Ikapitte et al., 2004) two IsoK/LGs react with the two amine groups of arginine to form bis-IsoK/LG adducts that then undergoes decomposition to a bis-IsoK/LG urea (BLU) adduct and ornithine

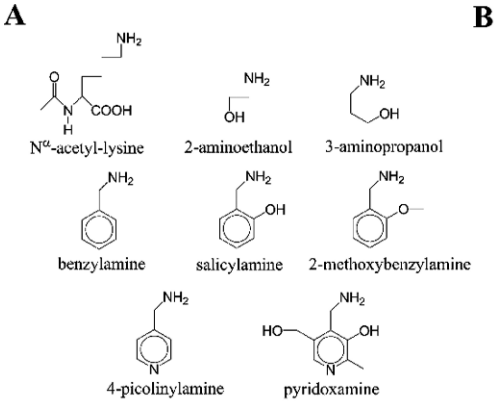
adduct and ornithine (Zagol-Ikapitte et al., 2004) (Fig. 2.4). To test the relative reactivity of IsoK/LG for lysine versus arginine, a polyglycine peptide also featuring one lysine and one arginine residue was reacted with 1 or 3 molar equivalents of IsoK/LG. When one molar equivalent of IsoK/LG was added, only the lysyl residue of the peptide was adducted. When three molar equivalents of IsoK/LG was added to the peptide, BLU adduct could be readily detected as well as the ornithine variant of the IsoK/LG lysyl adducted peptide. This result is consistent with the argininyl residue being substantially less reactive than the lysyl residue. The relevance of the reaction with arginine in vivo, especially its role in the formation of orthinine is currently under investigation.

Besides PE and arginine, other abundant endogenous primary amines include the polyamines. Polyamines such as spermidine and spermine have been postulated to protect DNA from reactive carbonyls and their high concentration in cytoplasm makes them a plausible agent for preventing the adduction of cytoplasmic proteins. Although polyamines have been demonstrated to be crosslinked to proteins in the presence of IsoK/LG (Boutaud et al., 2001), there have been no published characterizations of IsoK/LG-spermine or IsoK/LG-spermidine only adducts. If formed, these adducts might be useful surrogate markers for the extent of IsoK/LG formation under various oxidative conditions, as these adducts would not require proteolysis to be quantified by mass spectrometry making their measurement more straightforward than protein adducts.

2.1.4.2 Pyridoxamine Analogs are IsoK/LG Scavengers

That different primary amines reacted with IsoK/LG at different rates suggested the possibility that novel amines with even greater reactivity than lysyl residues could be identified. These novel amines could then be used as IsoK/LG scavengers to protect proteins from inactivation and to determine the impact on pathophysiology of specifically reducing the levels of IsoK/LG protein adduct. Screening a series of primary amines identified that pyridoxamine, a vitamin B_6 vitamer, potently competed with lysine for reaction to IsoK/LGs (Amarnath et al., 2004). To determine the structure-activity relationship for γ-ketoaldehyde scavenging, a series of pyridoxamine analogs were reacted with 4-oxopentanal (OPA), a model γ-ketoaldehyde, and the rate of pyrrole formation determined using Ehrlich reagent (Fig. 2.5). The second order reaction rate of pyridoxamine was found to be 2,309-fold faster than that of lysine. The key structural requirements for scavenging by pyridoxamine appear to be an aminomethyl group with an adjacent hydroxyl on an aromatic ring. Reaction of γ-ketoaldehyde with the aminomethyl group of pyridoxamine forms a hemiaminal adduct whose ketone group hydrogens bonds with the phenolic hydroxyl group of pyridoxamine. This bonding facilitates the nucleophilic attack on the ketone group required for ring closure and pyrrole formation. Methylation of the hydroxyl group prevents this hydrogen bonding and accounts for the highly diminished reactivity of 2-methoxybenzylamine compared to salicylamine.

The identification of the key features for IsoK/LG scavenging led to the synthesis of other related phenolic amines that also potently scavenge IsoK/LG in vitro such as pentyl-pyridoxamine (Davies et al., 2006). Lipophilic scavengers such as salicylamine and pentyl-pyridoxamine localize to the membranes where



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AMINE	Relative Reaction Rate
N^{α}-acetyl-lysine	1 x
2-aminoethanol	4 x
3-aminopropanol	5 x
benzylamine	11 x
salicylamine	983 x
2-methoxybenzylamine	9 x
4-picolinylamine	27 x
pyridoxamine	2309 x

Fig. 2.5 Structural requirements for IsoK/LG reactivity. Second order reaction rate of various pyridoxamine analogs for the formation of pyrrole adduct were determined relative to N^{α}-acetyl-lysine (Amarnath et al., 2004). The reaction rate of pyridoxamine was 2,309x faster than N^{α}-acetyl-lysine

IsoK/LG form and should therefore be more effective in vivo than hydrophilic compounds such as pyridoxamine. The greater efficacy of salicylamine and pentyl-pyridoxamine compared to pyridoxamine was borne out in studies where platelets were treated with individual scavengers and then activated with arachidonic acid to make LGs. While all three scavengers significantly inhibited the formation of LG protein adducts, salicylamine was the most potent followed by pentyl-pyridoxamine and then pyridoxamine (Davies et al., 2006).

How selective are pyridoxamine and lipophilic pyridoxamine analogs for scavenging γ-ketoaldehydes compared to other reactive lipid peroxidation products? The reactivity of pyridoxamine with α,β-unsaturated aldehydes is completely trivial, and pyridoxamine does not protect proteins from HNE adduction (Amarnath et al., 2004; Davies et al., 2006). Pyridoxamine does react with α-ketoaldehydes such as methylglyoxal that form from the oxidative decomposition of carbohydrates and lipids (Voziyan et al., 2002), and these adducts can be detected in the urine of rodents fed pyridoxamine in their drinking water (Metz et al., 2003). However, the reaction rate of pyridoxamine with γ-ketoaldehyde is 187 times greater than with methylglyoxal (Amarnath et al., 2004). To address the specificity of phenolic amine scavenging during lipid peroxidation, aliquots of lysine and iron-oxidized arachidonic acid were incubated with either pyridoxamine, pentyl-pyridoxamine, salicylamine, or vehicle and the resulting α- and γ-ketoaldehyde adducts measured by mass spectrometry (Davies et al., 2006). All three phenolic amines significantly reduced the levels of IsoK-lysyl-lactam adduct compared to vehicle. Levels of IsoK-phenolic amine adduct were nearly proportional to the decrease in lysyl-lactam adducts as would be expected for scavenging of IsoK. Importantly, IsoK-phenolic amine adduct were formed in significantly greater abundance than those derived from α-ketoaldehydes. Pentyl-pyridoxamine appeared to be most selective, with a nearly 50-fold greater yield of IsoK-pentyl-pyridoxamine adduct than the most abundant α-ketoaldehyde adduct. Salicylamine was the least selective phenolic amine with about a 7-fold greater abundance of IsoK-salicylamine adduct than the most abundant α-ketoaldehyde adduct. The discovery of these efficient and relatively selective IsoK/LG scavengers makes possible their future use in cells and in vivo to examine the contribution of IsoK/LG to cellular and organ dysfunction in the complex environment of oxidative stress.

2.2 Effects of IsoK/LGs on Cellular Function

2.2.1 IsoK/LG are Some of the Most Higly Cytotoxic Products of Lipid Peroxidation

Perhaps the most striking biological effect of IsoK/LG is their potent cytoxicity. The first demonstration of this effect came from direct injection of 100 nmol of

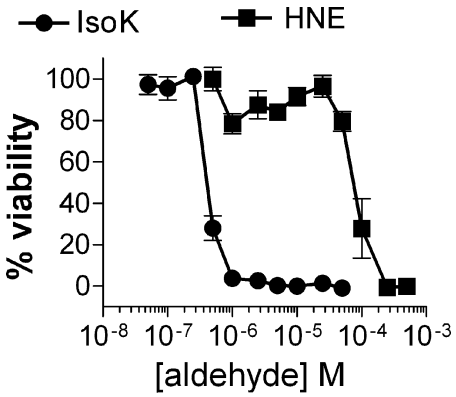

Fig. 2.6 IsoK is considerably more cytotoxic than HNE. P19 neuroglial cells were treated with various concentration of 15-E_2-IsoK or HNE for 24 h and the viability of cells determined by the MTT conversion assay. Percent viability was calculated relative to untreated cells. (Mean ± SEM $n = 8$)

IsoK/LG into the substance of the cerebral hemisphere of rats (Schmidley et al., 1992). The injected area showed pallor and loss of cellular constituents typical of necrotic cells and the marginal area became hypercellular because of the infiltration by macrophages. Evans Blue extravasation measurements also demonstrated a dose-dependent loss of blood brain barrier integrity. Adding nanomolar concentrations of IsoK/LG to cultured neuroglial cells directly induces cytotoxicity (Davies et al., 2002). IsoK/LG is several orders of magnitude more cytotoxic than HNE (Fig. 2.6), making it one of the most potent cytotoxic lipid peroxidation species known. Nevertheless, because toxicity in situ is a function of both the concentration of the product generated by peroxidation and of its potency, these experiments alone do not define to what extent each of the various peroxidation products contribute to the cytotoxicity induced by oxidative stress.

Because the IsoK/LG scavengers do not scavenge HNE and related electrophiles, these scavengers can be used to compare the contribution of IsoK/LG and HNE to the cytotoxicity of cells treated with hydrogen peroxide to induce oxidative stress. Pretreatment of HepG2 cells with IsoK/LG scavengers significantly inhibits the cytotoxicity induced by hydrogen peroxide (Fig. 2.7) (Davies et al., 2006). Only the lipophilic IsoK/LG scavengers, salicylamine and pentylpyridoxamine, provided protection, while the hydrophilic IsoK/LG scavenger, pyridoxamine, failed to provide significant protection. This result suggests that formation of IsoK adduct are a critical component of cell death induced by oxidative stress and that the critical targets of adduction are located near membranes, although lipophilic scavengers may also penetrate into the cells better than hydrophilic scavengers.

The exact mechanisms whereby IsoK/LG potently induces cell death are currently under investigation. IsoK/LG does not need to adduct to intracellular proteins to be toxic. Incubation of IsoK/LG with amyloid beta peptide causes oligimerization of amyloid beta (Davies et al., 2002) and IsoK/LG crosslinked amyloid β_{1-42} peptide is highly neurotoxic (Boutaud et al., 2006). IsoK/LG or

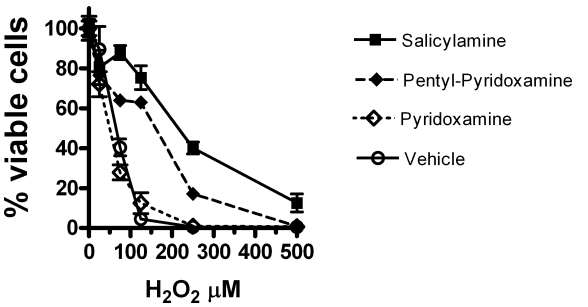

Fig. 2.7 Lipophilic IsoK/LG scavengers protect against cytotoxicity induced by oxidative stress. HepG2 cells were incubated with vehicle, pyridoxamine, pentyl-pyridoxamine, or salicylamine for 30 min prior to treatment with various concentrations of hydrogen peroxide (Davies et al., 2006). Viability was determined by detection of ATP using ATPlite luminescence assay and percent viability calculated relative to untreated cells (Mean ± SEM $n = 8$)

amyloid β_{1-42} alone did not produce similar neurotoxicity, so that adduction and oligimerization is required for this potent toxicity. Amyloid-derived diffusible ligands are thought to be critical neurotoxic products in Alzheimer's disease and antibodies to amyloid-derived diffusible ligands recognize IsoK/LG adducted amyloid β_{1-42}. Whether IsoK/LG contributes to the formation of amyloid-derived diffusible ligands in vivo remains to be determined. Another important question is whether the neurotoxic effect of IsoK/LG induced oligimerization can be generalized to other amyloid forming peptides.

2.2.2 Proteasome Inhibition may be an Important Mechanism of Cytotoxicity

The presence of oligimerized amyloid peptides does not, of course, account for the toxicity of IsoK/LG in culture cells that lack amyloid-like peptides. Nevertheless, studies with IsoK/LG adducted amyloid β may provide some insight into the generalized mechanisms that underlie IsoK/LG toxicity. For instance, IsoK/LG adducted amyloid β is a potent competitive inhibitor of the proteasome (Davies et al., 2002). The proteasome rids the cell of unneeded, incorrectly folded, or oxidatively damaged proteins. Pharmacological inhibition of the proteasome results in cell death, probably because of the accumulation of undesirable proteins. Proteolysis by the proteasome requires passing an unfolded loop of the protein down the barrel of the proteasome into the trypsin-, chymotrypsin-, and post-glutamyl peptidase-like catalytic sites buried deep within the multiple subunit complex. Incubating a model protein, ovalbumin, with equimolar IsoK/LG to form adducted protein decreased proteasomal degradation of the ovalbumin by about 50% (Davies et al., 2002). Incubating ovalbumin with ten molar equivalents of IsoK/LG completely prevented

proteasomal degradation. It should be noted that in these studies, the 20S proteasome was used rather than the 26S proteasome, so that blocking ubiquination by adducting the relevant lysines does not account for the decreased proteolysis. Instead, it is easy to imagine that adduction of lysine blocks the ability of the trypsin-like activity to hydrolyze the protein, so that the processing of IsoK/LG adducted proteins stalls inside the proteasome's barrel. These undigestable proteins would then inhibit access by other proteins targeted for degradation. Adduction of peptides with high affinity for the proteasome, such as amyloid β, would be particularly likely to inhibit proteasome activity. This mechanism is supported by a recent report that ubiquinated, IsoK/LG modified calpain-1 protein inhibited 26S proteasome activity while unmodified calpain-1 had no effect (Govindarajan et al., 2008). IsoK/LG can also directly act on the proteasome to inhibit its activity, although the exact mechanism for inhibition is unknown (Davies et al., 2002). Similarly, IsoK/LG can act on calpain-1, another significant pathway of protein degradation, to inhibit its activity (Govindarajan et al., 2008). Thus, formation of even a small amount of IsoK/LG or IsoK/LG adducted protein may lead to significant accumulation of undesired proteins within the cells and thus to cell death.

Accumulation of aggregated proteins is a hallmark of several neurodegenerative diseases, including Alzheimer's, Huntington's, and Parkinson's Disease. Proteasome activity, but not protein mass, is reduced in Alzheimer's disease. A recent report suggested that there is increased modification of proteasome by neuroketals in Alzheimer's disease brain (Cecarini et al., 2007). Although the specific epitope recognized by this antibody was poorly defined, if substantiated this finding would suggest proteasomal inhibition by IsoK/LG and neuroketal adducted proteins may very well play a central role in the neurodegeneration associated with these diseases.

2.2.3 IsoK/LG in Cardiovascular Disease

Another area of active interest into the effects of IsoK/LG is the role they may play in cardiovascular disease. While the increased levels of IsoK/LG adducts found in the circulation of atherosclerotic subjects suggests that formation of IsoK/LG may be important, this finding alone provides little insight into either the mechanisms of their formation or their role in the disease. Several plausible mechanisms may account for increased formation of IsoK/LG in atherosclerosis. Myeloperoxidase and cyclooxygenase levels increase in the vascular wall during atherosclerosis, and both enzymes generate prodigious amounts of IsoK/LGs, at least in vitro (Boutaud et al., 2001; Poliakov et al., 2003). Additionally, ischemia is well known to generate reactive oxygen species and ligation of the coronary descending artery in dogs for five days induced about a three-fold increase in the levels of IsoK/LG adducts, measured by mass spectrometry, in the infarct border zone (Fukuda et al., 2005). Using

immunohistochemistry, IsoK/LG adducts were found to localize to the epicardium and the myocardial core of the border zone after infarction.

2.2.3.1 IsoK/LG Adduction to Sodium Channel is Proarrhythmic

The localization of adducts to the epicardial border zone suggested the possibility that IsoK/LG adducts contribute to cardiac arrhythmias. Ventricular tachycardia/fibrillation following myocardial infarction is a major cause of sudden cardiac death. Arrhythmias in ischemic myocardium arise from sodium channel blockade. Sodium channels are hypothesized to cycle between three conformational states: a deactivated closed state, an activated open state, and an inactivated closed state. Upon depolarization, the deactivated state converts to the activated state and sodium current flows for a brief time before the channel enters the inactive state. The channel only converts from the inactive state to the deactivated state when the membrane repolarizes during the falling phase of the action potential. Changes in the ability to convert from the inactive to the deactivated state are critical to the initiation and perpetuation of arrhythmias.

Treatment of HEK-293 cells expressing the human cardiac sodium channel (Nav1.5-HEK) with an oxidant, tert-butyl-hydroperoxide (tBHP), results in a negative shift in the voltage dependence of inactivation (Fukuda et al., 2005). If translated in vivo, this negative shift would be proarrhythmic. In contrast to its effects on inactivation, tBHP treatment did not alter voltage dependent activation significantly. Thus oxidative stress does not simply destroy sodium channel function, but rather alters its susceptibility to inactivation. A similar effect of tBHP treatment occurs in HL-1 mouse atrial cell, which endogenously express both the cardiac sodium channel and its accessory beta subunits. Adding thiols such as DTT or glutathione, in order to scavenge α,β-unsaturated carbonyls such as HNE, did not protect sodium channel function during tBHP treatment. Additionally, treating Nav1.5-HEK cells with HNE did not induce voltage-dependent inactivation. Therefore, HNE and similar α,β-unsaturated carbonyls do not appear to contribute to sodium channel inactivation. In contrast to HNE, addition of 10 μM IsoK/LG to Nav1.5-HEK caused voltage-dependent inactivation of the sodium channel in a similar manner as tBHP. Addition of 15-F_{2t}-isoprostane, which is structurally related to IsoK/LG, but cannot adduct to proteins, had no effect on sodium channel function. IsoK/LG also caused sodium channel inactivation in HL-1 cells. Interestingly, treatment with IsoK/LG appeared to have overlapping as well as synergistic effects as flecainide, a Nav1.5 sodium channel blocker. An outstanding question is whether IsoK/LG scavengers blocked the effect of tBHP on the sodium channel. A preliminary conference report indicated that 10 μM salicylamine completely mitigated the effect of tBHP in Nav1.5-HEK cells and 100 μM salicylamine blocked the effect in HL-1 cells. If this report is substantiated, these results would strongly implicate IsoK/LG as important

effectors of sodium channel dysfunction after oxidative stress and would suggest the possibility that IsoK/LG scavengers might be effective antiarrhythmic agents. Studies are currently underway to test the efficacy of IsoK/LG scavengers in preventing arrhythmias after myocardial infarction in animal models. These studies should provide a clearer picture not only of the contribution of IsoK/LG to arrhythmias, but also to the efficacy of the scavengers in vivo.

2.2.3.2 Effect of IsoK/LG on Other Ion Channels

Besides the sodium channel, IsoK/LG has effects on other ion channels as well. For instance, addition of synthetic IsoK/LG to an atrial tumor myocyte cell line, AT-1, resulted in a pronounced dose-dependent inhibition of the inward rectifying potassium current induced by a –40 mV voltage step (Brame et al., 2004). Both activating and deactivating currents were suppressed, so the effect on potassium channels differs in this regard from sodium channels and suggests a more wholesale destruction of channel function. The IC_{50} for inhibition of I_{Kr} was 2.2 μM and full inhibition was only achieved after incubating the cells for 60 min. Washing the cells after this period of time did not revert I_{Kr} current to normal, consistent with covalent modification by IsoK/LG inducing the inhibition. The delayed time course of inactivation is consistent with the need to crosslink the channel in order to inactivate it, but no further attempt to characterize the mechanism of inhibition or the sites of adduction were reported.

Investigations into the effect of IsoK/LG modification on calcium channels or associated receptors that activate calcium release have only recently begun. Provocative preliminary results suggest that IsoK/LG modification may lead to activation of calcium currents, but additional experiments are needed to confirm this result and identify the mechanisms responsible.

2.2.3.3 Oxidized Lipoproteins

Although modifications of ion channels could contribute to the late stages of cardiovascular disease, formation of IsoK/LG adducts may also play a role early in atherosclerosis. Atherosclerosis is initiated when macrophages take up oxidized low density lipoprotein (oxLDL) via scavenger receptors such as SR-A and CD36 to form foam cells. In vitro oxidation of LDL results in the formation of IsoK adducts on the particle (Brame et al., 1999; Salomon et al., 1997a; 1999), suggesting that IsoK modification could potentially mediate this process. Addition of increasing concentration of IsoK/LG to native LDL results in a dose-dependent shift in the mobility of the LDL during electrophoresis, reminiscent of what is observed with oxLDL (Hoppe et al., 1997). Importantly, IsoK/LG modification of native LDL also dose-dependently increased the binding and uptake of the LDL by cultured mouse peritoneal macrophages to

a similar extent as found with oxLDL. Acetylated LDL, a substrate for SR-A, did not compete with IsoK/LG-modified LDL for uptake by these macrophages, but oxLDL completely competed off the binding and uptake of IsoK/LG-modified LDL. Therefore, IsoK/LG-modified LDL must be taken up by scavenger receptors other than SR-A. While these results suggest a role for IsoK/LG-modified LDL in atherosclerosis, they leave unanswered a number of important questions. For instance, do the receptors responsible for IsoK/LG-modified LDL uptake also facilitate the uptake of other IsoK/LG-modified proteins? Can IsoK/LG-modified LDL stimulate monocyte/macrophage chemotaxis and cytokine secretion in a similar manner as oxLDL? Hopefully, these and other questions will be addressed by future studies.

2.2.4 Effect of IsoK/LG on Other Generalized Cellular Functions

2.2.4.1 Tubulin/Microtubules

Microtubules play an integral part in a large number of cellular processes including mitosis. Polymerization and depolymization of tubulin, the major component of microtubules and one of the most abundant proteins in the cell, is critical to these functions and its polymerization is often regulated by membrane associated proteins. To test the effect of IsoK/LG adduction on microtubule function, IsoK/LG was added to sea urchin eggs. IsoK/LG dose-dependently (IC_{50} 15 μM) inhibited cell division of fertilized eggs, with lower doses (< 10 μM) inducing abnormal cleavage (Murthi et al., 1990). Whether similar inhibition of microtubule function occurs in mammalian cells is unclear as unpublished studies in cultured neuronal cells found that exogenous addition of IsoK/LG did not have significant effects either on neurite outgrowth or microtubule organization. Perhaps this is because insufficient IsoK/LG is able to penetrate through the membrane of neuronal cells, because incubation of purified GTP-depleted microtubule protein preparations from bovine brains with IsoK/LG dose-dependently inhibited microtubule assembly stimulated by addition of GTP (Murthi et al., 1990). Complete inhibition of tubulin polymerization required two molecules of IsoK/LG for each molecule of tubulin in the microtubule preparation. Interestingly, adduction of already assembled spindles by IsoK/LG did not lead to disassembly of the spindles. Similar concentrations of related lipid molecules, such as arachidonic acid or PGE_2 had no effect on microtubule assembly.

2.2.4.2 Histones and DNA

Addition of IsoK/LG to V79 Chinese hamster lung fibroblasts or nuclei caused formation of DNA-protein crosslinks (Murthi et al., 1993). These crosslinks were demonstrated indirectly by the amount of DNA retained on a nitrocelluse

filter after passing lysates from treated cells or nuclei through the filter. As the nitrocellulose filter normally traps only protein, but not DNA, the presence of DNA was indicative of a covalent bound with proteins. Treatment of the lysate with proteinase K prior to addition to the filter prevented DNA binding, confirming the requirement for covalent binding to protein. Formation of DNA-protein crosslinks was time-dependent, with 60 min or more required for maximal crosslinking, which is in keeping with the time required for formation of protein-protein crosslinks. The identity of the proteins involved in the DNA-protein crosslink was not determined. However, the lysine-rich histones readily undergo adduction in the presence of IsoK/LG (Boutaud et al., 2001) and DNA tightly coils around histones in cells, making these proteins the most likely protein candidate for DNA-IsoK/LG-protein crosslinks. What, if any, impact the formation of DNA-IsoK/LG-protein crosslinks has on the cell, or even if they form under biologically relevant conditions, has not been determined. However, it is not difficult to imagine that DNA-histone crosslinking might significantly disruption normal transcription and transcriptional regulation.

2.3 Concluding Remarks

Much of the early work relating to IsoK/LG has necessarily focused on understanding the basic chemistry of their reaction with proteins and other biological amines, developing methods to quantify IsoK/LG adducts in vivo, and developing selective inhibitors of their formation. From these initial studies comes tantalizing evidence for an important contribution of IsoK/LG adduction to pathophysiology. Not only are IsoK/LG adducts increased in disease conditions associated with oxidative stress and inflammation, but their application to cultured cells induce effects highly relevant to these conditions. The use of IsoK/LG scavengers improves cellular viability and function under oxidative stress. While these early results are encouraging, a large number of outstanding questions remain. For instance, are the levels of IsoK/LG modification measured in vivo sufficient to induce pathophysiology? Does treatment with IsoK/LG scavengers significantly lower the levels of IsoK/LG adducts in vivo and do these scavengers protect against disease? What are the mechanisms underlying IsoK/LG induced toxicity and ion channel dysfunction? Which specific proteins (or other biologically important amines) are adducted during oxidative stress and which ones are protected during treatment with IsoK/LG scavengers? Answering these questions may not only provide insight into the pathogenesis of diseases related to oxidative stress and inflammation, but also into the feasibility of using IsoK/LG scavengers as novel therapeutic agents for these conditions. Thus, the study of IsoK/LG may be fruitful field of endeavor for many years to come.

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