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FAST TRACK

# Carbonyl Compounds Cross-Link Cellular Proteins and Activate Protein-Tyrosine Kinase p60$^{c\text{-}Src}$

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**Abstract** Glyoxal, a dicarbonyl compound, is produced under oxidative stress by the autoxidation of glucose and reacts with the protein amino group to form Schiff base. In vitro treatment of murine thymocytes and fibroblasts with glyoxal induced extensive tyrosine phosphorylation of multiple proteins, which was drastically inhibited by the addition of OPB-9195, an inhibitor of the carbonyl reaction with proteins. Glyoxal induced cross-linking of a number of cellular proteins, including glycosylphosphatidylinositol (GPI)-anchored cell surface Thy-1. We then demonstrated that treatment of cells with glyoxal promptly induced activation of non-receptor protein-tyrosine kinase c-Src, which was partially inhibited by OPB-9195. It is suggested from these results that carbonyl amine reaction quickly activates c-Src, possibly through cross-linkage of GPI-anchored proteins or putative specific receptors. J. Cell. Biochem. 72:1–7, 1999. 

**Key words:** glyoxal; tyrosine phosphorylation; Thy-1; c-Src

The autoxidation of glucose under oxidative stress leads to the production of dicarbonyl glyoxal [Wells-Knecht et al., 1995], which might mediate diseases through a complex reaction pathway. Initially a condensation reaction occurs between its highly reactive carbonyl ($-C = O$) and protein amino ($-NH_2$) group to form Schiff base, which undergoes subsequent rearrangement and dehydration reactions to ultimately form stable end products called the advanced glycation end products (AGEs) such as $N^{\epsilon}$-carboxymethyllysine (CML) [Wells-Knecht et al., 1995; Glomb and Monnier, 1995]. The latter process requires at least several hours for completion after the initial formation of Schiff base. Using immunohistochemical and chemical methods, different study groups have demonstrated the progressive accumulation of AGEs in tissue proteins in aging [Monnier, 1990; Horiuchi and Araki, 1994; Fu et al., 1994; Jiaan et al., 1995], diabetic complications [Fu et al., 1994; Miyata and Monnier, 1992; Nakamura et al., 1993], uremic complications such as dialysis-related amyloidosis [Miyata et al., 1993, 1996a], and atherosclerotic lesions [Uchida et al., 1997].

Highly reactive carbonyl may directly cause damage of cells and tissues, but the mechanism of carbonyl-mediated tissue damage still remains largely unknown. AGEs have been shown to interact with a distinct class of cellular receptors [Neeper et al., 1992; Hori et al., 1996], thereby generating an oxidant stress capable of altering gene expression and cellular properties [Yan et al., 1994; Miyata et al., 1996b]. More recently it has been shown that binding of AGEs to their receptors triggers a cascade of intracellular signals involving activation of $p21^{ras}$ and mitogen-activated protein kinase (MAPK) [Lander et al., 1997]. It is, however, unclear whether the initial step of carbonyl amine reaction for Schiff base formation before AGE production delivers some signals intracellularly.

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By analyzing the mechanism of activation of lymphocytes with sulfhydryl (SH)-group–reactive heavy metal ($HgCl_2$), we previously proposed a novel mechanism of receptor activation replacing physiological non-covalent binding between ligand and receptors with protein-S-Hg-S-protein bond–mediated aggregation of multiple cell surface proteins/receptors [Nakashima et al., 1994]. We here have tested a hypothetical view that Schiff base formation between highly reactive carbonyl and protein amino group on cells as an initial step for AGE formation would activate cell surface receptors for signal transduction. The results show that at an early time before production of AGEs glyoxal delivers signals for multiple cellular protein-tyrosine phosphorylation in murine thymocytes and fibroblasts and activation of non-receptor protein tyrosine kinase (PTK) $p60^{c\text{-}Src}$ (c-Src), possibly through a chemical activation of cell surface receptors.

## MATERIALS AND METHODS

### Cells and Reagents

Single cell suspensions of thymocytes in Eagle's minimum essential medium (MEM) were prepared from 6- to 8-week-old C57BL/6 strain mice. They were incubated in the presence or absence of glyoxal (Sigma, St. Louis, MO), at 37°C before analysis. The murine NIH3T3 fibroblast cell line overexpressing c-Src kinase was kindly provided by Dr. D. Shalloway, Pennsylvania State University. The NIH3T3 cell lines were cultured in plastic plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% $CO_2$/95% air incubator. After becoming confluent, cells were collected with 0.25% trypsin, 0.01% EDTA in phosphate-buffered saline and were split into 60-mm plastic plates with DMEM containing 10% FCS for a further 20- to 24-h incubation. The cells were then rinsed with fresh MEM twice and incubated in MEM at 37°C for 1 h before stimulation with glyoxal for analysis.

### Electrophoresis and Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot were performed as described elsewhere [Nakashima et al., 1994]. Briefly, cells were lysed by adding SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol), and proteins thus obtained were subjected to SDS-PAGE with 10% gel. The gel was stained with Coomassie brilliant blue (R-250), or the proteins in the gel were transferred to polyvinylidene difluoride membrane. The membrane was incubated with anti-phosphotyrosine (Ptyr) affinity-purified polyclonal antibody (Transduction Lab., Lexington, KY) or with anti-Src monoclonal antibody (mAb327, donated by Dr. J.S. Brugge, State University of New York) [Lipsich et al., 1983] overnight at 4°C, and then with the appropriate second antibody for 2 h at room temperature. The proteins were visualized by Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA) as directed by the manufacturer. The molecular sizes of the developed proteins were estimated by comparison with prestained protein markers (New England Biolabs, Beverly, MA).

### Fluorescence Antibody Technique

Thymocytes ($10^6$/100 µl) were incubated with or without glyoxal (50 mM) at 37°C for 10 min to 1 h and were fixed with 4% paraformaldehyde at room temperature for 30 min. They were then stained with fluorescein isothiocyanate (FITC)-labeled anti-Thy-1.2 monoclonal antibody (Becton Dickinson, Mountain View, CA) and were mounted on a glass slide in the presence of p-phenylenediamine (1 mg/ml). The stained cells were observed under a fluorescence microscope.

### In Vitro Kinase Assay

Cells were lysed with 1.0 ml of ice-cold RIPA buffer (10 ml Tris-HCl, pH 8.0, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.5 mM $Na_3VO_4$, and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 15,000 rpm for 20 min at 4°C, and anti-Src monoclonal antibody (mAb327) was added to the supernatant. The immunoprecipitates were collected by incubating with protein A sepharose beads (Pierce, Rockford, IL) which had been conjugated with rabbit anti-mouse IgG antibody (MBL, Nagoya, Japan). The reaction mixture for the kinase assay was prepared by adding 1.5 µg acid-denatured enolase (Sigma) as an exogenous substrate, 370 kBq [$\gamma$-$^{32}$P]ATP (NEN, Wilmington, DE), and kinase buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) with a final volume of 30 µl. The kinase reaction was carried out for 20 min at 30°C and was stopped by adding 30 µl 2× SDS-sample buffer with 2-ME. The immuno-

precipitates were then heated in a boiling water bath for 3 min, and phosphoproteins were analyzed on 10% SDS polyacrylamide gels. Gels were dried and exposed to X-ray film at −80°C for autoradiography.

## RESULTS AND DISCUSSION

We first investigated whether a short-term exposure of murine thymocytes to a highly reactive carbonyl-containing chemical, glyoxal, could transduce signals for phosphorylating cellular proteins at the tyrosine residue. As shown in Figure 1, exposure of cells to 10–50 mM glyoxal induced tyrosine phosphorylation of a number of cellular proteins in a concentration-dependent manner. The molecular weights of the proteins phosphorylated were 200, 120, 80/75, 60/56, 42/40, and 28 kDa. Another dicarbonyl compound, malondialdehyde, which is structurally similar to glyoxal but contains one additional carbon, also induced tyrosine phosphorylation of proteins at lower concentrations (2–10 mM) (data not shown).

A thiazolidine derivative, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195, developed by Fuji Memorial Research Institute, Otsuka Pharmaceutical, Ohtsu, Japan), has recently been shown to interact with carbonyl groups and thereby to inhibit formation of AGEs by trapping the carbonyls to prevent Schiff base formation between the carbonyl compound and the protein as the initial event of AGE formation [Nakamura et al., 1997] (Miyata et al., unpublished data). We tested whether OPB-9195 could affect the glyoxal-mediated signal that might be generated after Schiff base formation with cellular proteins. As shown in Figure 1B, addition of OPB-9195 prior to glyoxal into the suspension of thymocytes drastically inhibited the induction of protein-tyrosine phosphorylation. This suggested that the formation of carbonyl-mediated Schiff base with proteins is crucial for generation of the signal for intracellular protein-tyrosine phosphorylation.

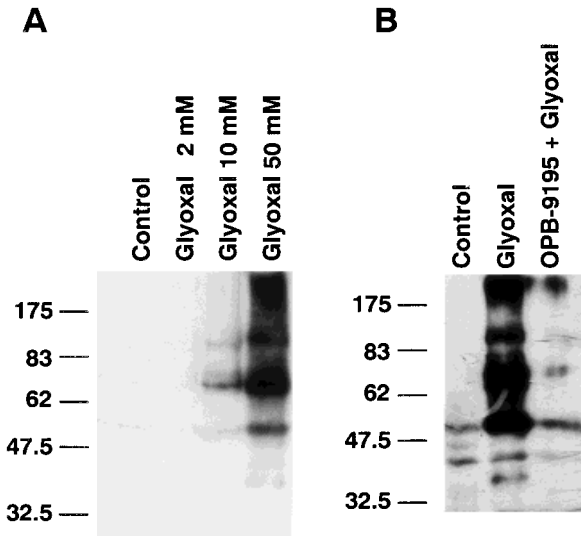


**Fig. 1.** Glyoxal induces tyrosine phosphorylation of cellular proteins. Thymocytes ($10^7$/100 µl) were incubated at 37°C with or without the indicated concentrations (**A**) or 50 mM (**B**) of glyoxal for 10 min. One group of cells from B was preincubated with 1 mM OPB-9195 for 10 min prior to addition of glyoxal. The cells were then lysed with sample buffer and subjected to immunoblot assay with anti-Ptyr antibody. Positions of molecular mass markers (kDa) are shown on the left.

The time-course study for the glyoxal effect on thymocytes showed that protein phosphorylation started after 2 min of stimulation and reached a peak after 10 min (Fig. 2A-1). When the incubation time increased further, the phosphorylation of lower-molecular-weight proteins gradually decreased, whereas that of higher-molecular-weight proteins increased. After a 1-h incubation, a large amount of phosphorylated proteins was detected at the site of the membrane corresponding to the upper region of both stacking and separating gels. From this result it was predicted that during a 1-h incubation, glyoxal cross-linked many Ptyr-containing proteins or proteins that associate with the phospho-proteins to form aggregates which did not run through the separating gel. Some of the aggregates became so large that they even did not move through the stacking gel. Coomassie blue staining of the gel for total cellular proteins confirmed the formation of aggregates of proteins at the loading gel region, the amount of which increased with time (Fig. 2A-2). A similar time-course effect of glyoxal was also demonstrated on NIH3T3 cells that overexpressed c-Src kinase. In NIH3T3 cells protein phosphorylation, however, advanced more slowly than in thymocytes. When the cells were incubated for 30 min or more, a number of cellular proteins were phosphorylated extensively, and some Ptyr-containing proteins (Fig. 2B-1) and proteins stained with Coomassie blue (Fib. 2B-2) were detected at the site of the membrane corresponding to the upper region of both stacking and separating gels.

When cell viability was examined by staining the cells with Trypan blue, >95% of NIH3T3

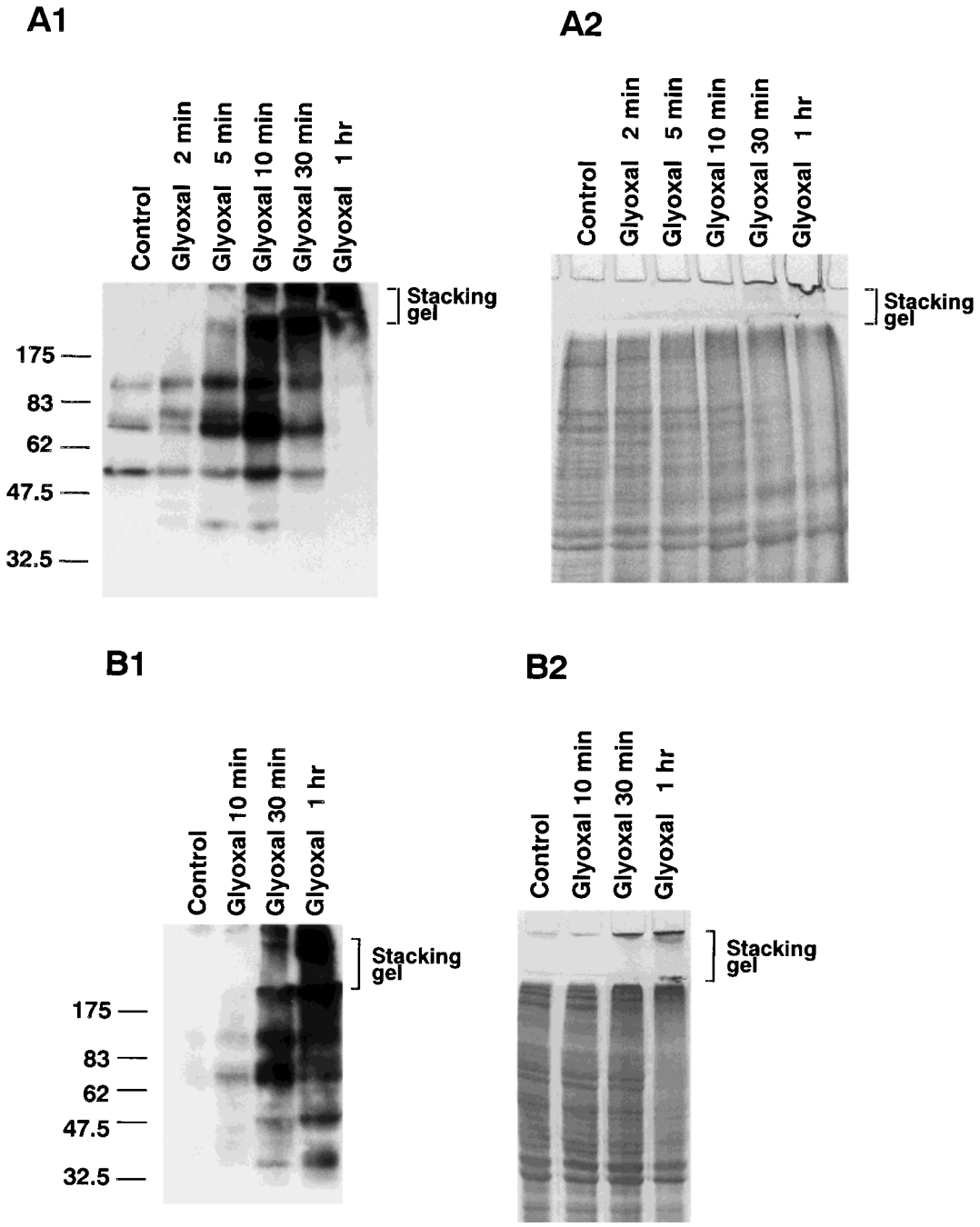


**Fig. 2.** Time course of glyoxal-induced protein-tyrosine phosphorylation and aggregation of cellular proteins. Thymocytes (**A:** $10^7$/100 μl) and NIH3T3 cells overexpressing c-Src (**B:** confluent) were incubated at 37°C with or without 50 mM glyoxal for the indicated time. The cells were then lysed with sample buffer and subjected to SDS-PAGE for immunoblot assay with anti-Ptyr antibody (**A1,B1**). The gels after SDS-PAGE were also stained with Coomassie blue (**A2,B2**). Positions of molecular mass markers (kDa) are shown on the left.

cells and >70% of thymocytes were alive after a 1-h incubation with 50 mM of glyoxal. This suggested that cell surface proteins on live cells may be the primary target of glyoxal for Schiff base formation. We examined whether some cell surface proteins such as glycosylphosphatidylinositol (GPI)-anchored Thy-1, as an example, would be cross-linked for aggregation. We stained glyoxal-treated and -untreated thymocytes with FITC-labeled anti-Thy-1 antibody and observed them under a microscope. As shown in Figure 3, glyoxal treatment induced

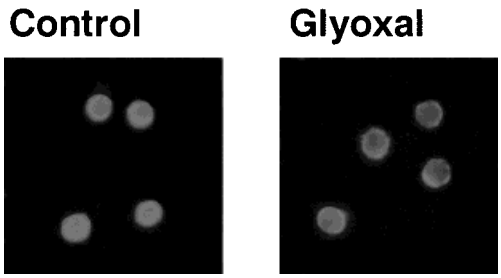


**Fig. 3.** Glyoxal induces aggregation of Thy-1 on thymocytes. Thymocytes ($10^6$/100 µl) were incubated at 37°C with or without 50 mM of glyoxal for 10 min to 1 h. They were then fixed with paraformaldehyde and were stained with FITC-labeled anti-Thy-1 mAb. Fluorescence was examined under a fluorescence microscope. Shown are representatives of more than 100 photographs (×400) obtained by four independent experiments.

aggregation of cell surface Thy-1 proteins as revealed by localized fluorescence. Aggregation of Thy-1 first developed at 10 min after the treatment and progressed further with increasing time.

Promotion of tyrosine phosphorylation of cellular proteins may be caused by either activation of some PTKs or inactivation of protein tyrosine phosphatases. We then asked whether carbonyl amine reaction would induce activation of PTKs and examined the effect of exposure of glyoxal to NIH3T3 cells overexpressing c-Src, which are best suited for monitoring any fine changes in the kinase activity [Pu et al., 1996]. c-Src, one of non-receptor type PTKs, is widely distributed in various cell types and is potentially involved in the signal delivery for controlling cellular growth and function [Cantley et al., 1991; Cooper and Howell, 1993]. As shown in Figure 4, treatment of the NIH3T3 cells with glyoxal induced activation of Src kinase as revealed by both enhanced autophosphorylation of c-Src kinase and phosphorylation of the exogenous substrate enolase. Compared to no treatment control, cells incubated in the presence of 10–50 mM glyoxal displayed augmented c-Src kinase activity in a concentration-dependent manner (Fig. 4A-1). The time-course study in the presence of 50 mM glyoxal showed that the kinase activity was elevated within 10 min incubation and was increased further with increasing time, reaching a peak at 1 h (data not shown). We then tested whether OPB-9195 could affect glyoxal-mediated kinase activity. OPB-9195 partially but definitely inhibited the glyoxal-mediated activation of c-Src kinase (Fig. 4A-2). Whereas

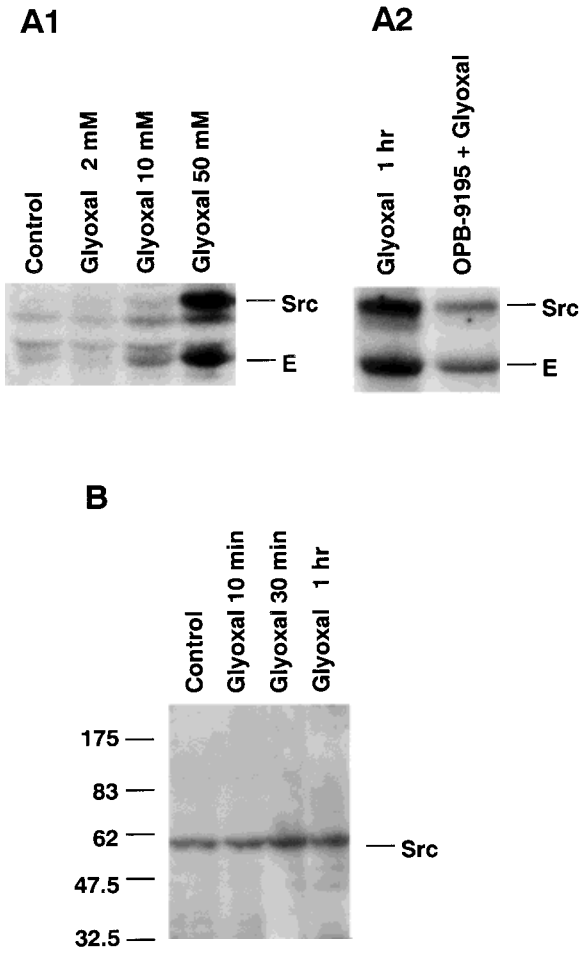


**Fig. 4.** Glyoxal induces autophosphorylation and activation of c-Src kinase. NIH3T3 cells were incubated at 37°C with or without indicated concentrations of glyoxal for 10 min (**A1**) or 50 mM of glyoxal for indicated time (**A2,B**). The cells of the right lane of A2 were preincubated with 1 mM OPB-9195 for 10 min prior to the addition of glyoxal, followed by a further 1-h incubation with glyoxal. The cells were lysed with RIPA buffer and subjected to immunoprecipitation with anti-Src antibody for in vitro kinase assay (A), or lysed with sample buffer for immunoblotting with anti-Src antibody (B). Positions of molecular-mass markers are shown on the left, and those of the Src protein (Src) and enolase (E) on the right.

some cellular proteins were shown to be aggregated by glyoxal (see Fig. 2), the heavily autophosphorylated c-Src proteins were localized at the original position in the SDS gel with no evidence of aggregation of the c-Src proteins (Fig. 4A). Examination by Western blot with anti-Src antibody also ruled out the possibility that Src proteins themselves were aggregated for activation by the reaction with glyoxal (Fig. 4B). Src family PTKs are known to associate across plasma membrane with both transmembrane and GPI-anchored cell surface proteins/

receptors [Stefanova et al., 1991; Thomas and Semelson, 1992; Shenoy-Scaria et al., 1992], crosslinking of which could activate the kinases [Nakashima et al., 1991, 1993]. Although no specific transmembrane receptor for c-Src has been identified, it could be that intracellular c-Src kinase was activated through Schiff base formation-mediated cross-linkage of GPI anchored proteins or putative transmembrane receptors.

The concentration of glyoxal that was effective for signal transduction (10–50 mM) might be too high to be naturally attained in the physiological environment of the cell. High concentrations of glyoxal (20–200 mM) were, however, used in vitro under physiological environment for demonstrating polymerization of RNAse by cross-linking [Glomb and Monnier, 1995]. Moreover, the unexpectedly low cytotoxicity of the high concentration (50 mM) of glyoxal in our experiment may suggest a rapid decay of the glyoxal in the physiological environment of cells before reacting with critical cellular proteins for signal transduction and cytotoxicity. This view may also explain the reason why the signal transducing effect of high concentration (50 mM) of glyoxal was inhibited by low concentration (1 mM) of OPB-9195.

Glyoxal can be produced in vivo when glucose or polyunsaturated fatty acid such as arachidonic acid is autoxidized [Wells-Knetcht et al., 1995; Fu et al., 1996] and has been detected on tissues of a number of stress-linked diseases [Uchida et al., 1997]. Production of glyoxal in vivo under physiological condition can yield AGEs such as CML, which have been reported to accumulate in long-lived proteins with age and in diabetic patients [Fu et al., 1994]. Our present results suggest that the demonstrated new mechanism for intracellular signal transduction is at least in part involved in the pathogenesis of these diseases at an early stage.

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