Lysine N-pyrrolation converts lysine residues to N\(^{+}\)-pyrrole-L-lysine (pyrK) in a covalent modification reaction that significantly affects the chemical properties of proteins, causing them to mimic DNA. pyrK in proteins has been detected in vivo, indicating that pyrrolation occurs as an endogenous reaction. However, the source of pyrK remains unknown. In this study, on the basis of our observation in vitro that pyrK is present in oxidized low-density lipoprotein and in modified proteins with oxidized polyunsaturated fatty acids, we used LC–electrospray ionization–MS/MS coupled with a stable isotope dilution method to perform activity-guided separation of active molecules in oxidized lipids and identified glycolaldehyde (GA) as a pyrK source. The results from mechanistic experiments to study GA-mediated lysine N-pyrrolation suggested that the reactions might include GA oxidation, generating the dialdehyde glyoxal, followed by condensation reactions of lysine amino groups with GA and glyoxal. We also studied the functional significance of GA-mediated lysine N-pyrrolation in proteins and found that GA-modified proteins are recognized by apolipoprotein E, a binding target of pyrrolated proteins. Moreover, GA-modified proteins triggered an immune response to pyrrolated proteins, and monoclonal antibodies generated from mice immunized with GA-modified proteins specifically recognized pyrrolated proteins. These findings reveal that GA is an endogenous source of DNA-mimicking pyrrolated proteins and may provide mechanistic insights relevant for innate and autoimmune responses associated with glucose metabolism and oxidative stress.

Covalent modification of lysine residues by electrophilic aldehydes gives rise to formation of heterocyclic structural motifs such as pyroles, pyridines, and pyrazines. The pyrrole framework is particularly interesting because of its chemical properties and presence in a wide variety of biologically active natural compounds (1). Recently, a covalent modification reaction of proteins, converting lysine residues to N\(^{+}\)-pyrrole-L-lysine (pyrK), was discovered by Miyashita et al. (2). Lysine N-pyrrolation (Fig. 1A) is associated with an increase in net negative charges of proteins because of formation of a lysine charge-neutralizing pyrrole structure. More interestingly, proteins gain electrical conductivity via pyrrolation (2). The mechanisms underlying these electronic properties have been suggested to be due to stacking interactions between the pyrrole rings and/or between pyrrole ring and aromatic amino acid residues. Because of these properties, pyrrolated proteins have been claimed to be DNA-mimicking molecules. Indeed, it has been shown that DNA-binding molecules, such as anti-DNA autoantibodies and DNA intercalators, recognize pyrrolated proteins and that immunization of mice with pyrrolated proteins triggers enhanced production of anti-DNA autoantibodies (2). Using a highly sensitive and specific LC-MS/MS–based method, pyrK has been detected in immune complex deposits in kidneys of mice (2), suggesting involvement of a physiologically relevant molecule in lysine N-pyrrolation.

On the other hand, Hirose et al. (3) recently identified apolipoprotein E (apoE) as an innate pyrrole-binding protein in human serum that promotes uptake of pyrrolated proteins by macrophages. They have also shown that deficiency of apoE leads to a significant elevation of pyrrolated proteins (3). In addition, significantly elevated levels of serum pyrrolated proteins compared with normal control individuals have also been demonstrated. These findings support our proposition that lysine N-pyrrolation in proteins, generating pyrK, occurs in vivo and provide a link connecting lysine N-pyrrolation and lipoprotein metabolism. However, the key question is what mediates N-pyrrolation. 1,4-Butanediol (BDA), a Paal–Knorr reagent, can readily convert lysine to pyrK, but it is not a physiological molecule. Several studies have shown that conversion of primary amino groups into pyrrole derivatives occurs upon reaction of lysine residues of proteins with the reactive species that originated from the lipid peroxidation reactions. They are mostly aldehydes, such as 4-hydroxy-2-nonenal (4, 5), 4-oxo-2-alkenals (6), 4,5-epoxy-2-alkenals (7), levuglandin E2 (8), and hydroxy-\(\omega\)-oxoalkenoic acids (9). Unsaturated epoxyxoxy fatty acids have also been reported to generate pyrrole derivatives on lysine residues (10). In our previous study, we demonstrated that oxidative modification of LDL, generating a number of lipid peroxidation-derived aldehydes, was accompanied by an increase in binding potential of the oxidized LDL with a DNA intercalator. Based on these observations, we tentatively con-
cluded that pyrK was being formed from a product of oxidation of the lipid component of LDL (2). Moreover, given the structure of the pyrrole ring in pyrK, the most likely candidates were anticipated to be short-chain aldehydes, especially C2 and/or C4 aldehydes.

To understand the potential physiological and pathophysiological significance of lysine N-pyrrolation, we initiated studies to identify pyrrolating agent(s) from in vitro peroxidized polyunsaturated fatty acids (PUFAs) and unequivocally identified a C2 aldehyde, glycolaldehyde (GA), as a source of pyrK. We also characterized the mechanism of GA-mediated lysine N-pyrro-lation and suggest that the reaction might require oxidation of GA. Following identification of GA as an active substance, we examined the interaction between GA-modified proteins and apoE, a binding target of pyrrolated proteins. We also generated several monoclonal antibodies from mice immunized with GA-modified proteins and revealed their specificity toward pyrrolated proteins. Our findings uncovered a novel function of GA as an endogenous source of DNA-mimicking pyrrolated proteins and suggest that GA-derived pyrK residues generated in proteins may play a role in innate and autoimmune responses associated with glucose metabolism and oxidative stress.

Results

Involvement of lipid peroxidation in lysine N-pyrrolation

To obtain direct evidence of the occurrence of lysine N-pyrrolation in oxidized LDL, we analyzed pyrK in Cu2+-oxidized LDL using LC-ESI-MS/MS coupled with a stable isotope dilution method. When isolated human plasma LDL (0.5 mg/ml) was incubated at 37 °C with Cu2+ (5 μM), the yield of pyrK in low-density lipoproteins increased in a time-dependent manner (Fig. 1B). The yield of the adduct was 0.53 mol/mol of LDL after 24 h of incubation. The data strongly suggest that lipid peroxidation is involved in formation of pyrK in oxidized LDL.

Then, based on the fact that pyrrolated proteins act as DNA mimics recognized by SYBR Green I (SG), we carried out activity-guided separation of oxidized fatty acids upon production of SG–binding proteins. Oxidized PUFAs (EPA and DHA) were separated into aqueous and organic fractions and determined for production of SG-stainable proteins upon incubation with BSA at 37 °C for 48 h. Electrophoresis was performed in a nondenaturing polyacrylamide gel. After electrophoresis, the gels were stained with CBB (left panel) and SG (right panel).

Identification of a pyrrolating agent generated during peroxidation of PUFAs

To identify the oxidized lipid(s) responsible for formation of pyrK in the protein, the aqueous fractions of oxidized EPA and DHA were separated and fractionated every 1 min by reverse-phase HPLC. Aliquots of each fraction were then incubated with BSA in 50 mM phosphate buffer (pH 7.2) at 37 °C for 4 days, and their activities regarding production of SG-binding proteins were evaluated. It appeared that multiple fractions in oxidized EPA and DHA could generate SG-stainable proteins (Fig. 2). We tested these fractions for formation of pyrK upon incubation with BSA and observed that only fraction 4 commonly produced pyrK. The data suggest that the same molecule origi-
An endogenous source of lysine N-pyrrolation

Figure 2. Presence of pyrrolating agent(s) in oxidized PUFAs. A, HPLC fractions for production of SG-stainable proteins. Top panel, oxidized EPA. Bottom panel, oxidized DHA. Lipid peroxidation was performed by incubating 12.5 mM PUFA ethyl esters (EPA and DHA) in the presence of 200 μM Cu²⁺ and 10 mM H₂O₂ in 200 μl of 50 mM sodium phosphate buffer (pH 7.2) in atmospheric oxygen at 37 °C. After 24 h, the reaction mixture was extracted with an equal volume of chloroform:methanol (2:1) and vortexed. After centrifugation, the upper (aqueous) phase was collected. The aqueous phases of the oxidized PUFA samples were subjected to reverse-phase HPLC, and the eluates were collected at 1-min intervals. The collected samples were evaporated and incubated with 1 mg/ml BSA at 37 °C for 4 days, and production of SG-stainable proteins was determined by measuring the fluorescence (excitation/emission at 494/521 nm) using a micro-ELISA plate reader. Solid lines, profile of UV absorbance at 220 nm; columns, SG fluorescence. Fr. 4, fraction 4. B, measurement of pyrK generated upon incubation of BSA with fraction 4. After alkaline hydrolysis of native and modified BSAs, pyrK was measured by LC-ESI-MS/MS. Top panel, standard pyrK. Center panel, measurement of pyrK generated in the protein incubated with fraction 4 from the oxidized EPA. Bottom panel, measurement of pyrK generated in the protein incubated with fraction 4 from the oxidized DHA.

Peroxidation of PUFA produces complex breakdown products, including alcohols, aldehydes, ketones, and ethers. We speculated that the most likely sources of pyrK might be aldehydes. Hence, fraction 4 from oxidized EPA and DHA was incubated with 2,4-dinitrophenylhydrazine (DNPH) and analyzed by reverse-phase HPLC. As shown in Fig. 3A, the fractions gave the same product (indicated by arrows). To identify the putative pyrrolating agent, the DNPH derivatives of fraction 4 from oxidized EPA and DHA were further analyzed by LC-ESI-MS. Each fraction gave two common peaks (peaks a and b from oxidized EPA and peaks c and d from oxidized DPA) at 4.6 min and 4.9 min (Fig. 3, B and C). Peaks a (Fig. 3B, center panel) and c (Fig. 3C, center panel) showed an [M-H]⁻ peak at m/z 197 corresponding to the m/z of the unreacted DNPH. Peaks b (Fig. 3B, bottom panel) and d (Fig. 3C, bottom panel) gave the same [M-H]⁻ ion at m/z 239, which was expected to be the DNPH derivative of the putative pyrrolating agent that originated from the PUFA samples. Because DNPH derivatization of an aldehyde is an addition reaction followed by dehydration, the original pyrrolating agent was expected to have a molecular mass of 60 Da. Considering that it has an aldehyde moiety that originated from lipid peroxidation, the most likely structure of this product was GA (Fig. 3D). The DNPH derivative of the authentic GA (Fig. 3E) indeed gave the same fragmentation pattern as the products that originated from the oxidized PUFA samples (Fig. 3, B and C). In addition, we also confirmed that authentic GA chromatographed with the activity in fraction 4.

Using the stable isotope dilution–based LC-ESI-MS/MS method, we confirmed that a significant amount of GA could be generated during peroxidation of the PUFA samples (Fig. S1 and Fig. 4). Thus, GA appeared to be a putative pyrrolating agent generated during peroxidation of the PUFA.

GA mediates lysine N-pyrrolation of proteins

To establish that GA could mediate lysine N-pyrrolation in proteins, we examined production of SG-stainable DNA mimic proteins and pyrK upon incubation of GA with BSA. As shown in Fig. 5, A and B, GA indeed produced modified proteins that could be recognized by the DNA intercalator. The GA-modified proteins also showed significant cross-reactivity with an anti-DNA IgM mAb, ADL19 (Fig. 5C). In parallel with production of DNA mimic proteins, a significant amount of pyrK was detected in GA–modified proteins (Fig. 5D). Thus, GA was identified as the first endogenous metabolite that could mediate lysine N-pyrrolation.

To examine whether any other endogenous metabolites could be involved in lysine N-pyrrolation, we tested several carbohydrates and their related metabolites with a chain length between two and four carbon atoms. We also tested C5 and C6 reducing sugars that could serve as their precursors. As shown in Fig. 6A, in addition to GA, glycolytic intermediates, such as glyceraldehyde and methylglyoxal, generated DNA-mimicking.
SG-stainable proteins. In addition, the SG-binding potency of these modified proteins correlated well with their ability to bind to the anti-DNA IgM mAb ADL19 (Fig. 6B). Thus, glyceraldehyde and methylglyoxal, in addition to GA, were tentatively identified as the most likely candidates of physiological pyrrolating agents. To determine whether the SG-binding potency could be ascribed to N-pyrrolation of lysine residues, we measured pyrK in modified proteins using LC-ESI-MS/MS coupled with a stable isotope dilution method. Interestingly, pyrK was primarily detected in protein modified with GA (Fig. 6C). Despite significant SG-binding potency, methylglyoxal was hardly involved in formation of pyrK upon incubation with the protein and the lysine derivative. It is likely that methylglyoxal may generate a pyrK-like adduct, which might contribute to recognition of modified proteins by SG.

**Involvement of oxidized GA**

Lysine N-pyrrolation by GA may be explained by a mechanism involving formation of a Schiff base derivative as the first intermediate. The Schiff base further reacts with a second GA molecule to generate an imine derivative, which is subsequently converted to the final product (pyrK). Theoretically, conver-
Two sets of nucleophilic addition reactions, pyrK involving GA and glyoxal. The mechanism may include pyrrolated proteins and showed that the apoE deficiency may, at least in part, be involved in lysine oxidation (Fig. 7B). Thus, oxidative conversion of GA to glyoxal could mediate lysine N-pyrrolation upon incubation with NAK. pyrNAK was barely produced by glyoxal alone; however, pyrrolation was dramatically accelerated by combination of GA and glyoxal (Fig. 7B). The data suggest involvement of oxidation of a GA–lysine intermediate and/or oxidation of GA itself. In this regard, based on the previous finding that GA can be easily oxidized to glyoxal (11), we examined whether oxidized GA–lysine intermediate could mediate lysine N-pyrrolation upon incubation with NAK. pyrNAK was barely produced by glyoxal alone; however, pyrrolation was dramatically accelerated by combination of GA and glyoxal (Fig. 7B). Thus, oxidative conversion of GA to glyoxal may, at least in part, be involved in lysine N-pyrrolation by GA (Fig. 7C). Fig. 8 depicts a proposed mechanism for formation of pyrK involving GA and glyoxal. The mechanism may include two sets of nucleophilic addition reactions, i.e. between a primary amino group of the lysine residue and GA and between a secondary amine derivative (Amadori product) and glyoxal.

**GA-modified proteins act as a ligand of apoE**

We recently identified apoE as a binding target of pyrK and pyrrolated proteins and showed that the apoE deficiency leads to significant accumulation of pyrrolated serum proteins and an enhanced immune response (3). Hence, to determine whether GA-modified proteins could show affinity for serum proteins, including apoE, normal mouse serum was incubated with beads coupled to native BSA and GA-modified BSA (GA-BSA). We used two GA-BSA ligands that differ depending on the incubation period (1 and 7 days). The bound proteins were eluted and separated by SDS-PAGE under reducing conditions (Fig. 9A). Several unique bands were detected in pulldown with the GA-BSA ligands (Tables S1–S7). Among them, a protein with a molecular mass of about 35 kDa (Fig. 9A, band 6) that was pulled down by GA-BSA was identified as apoE by LC-MS/MS analysis. Another six proteins (Fig. 9A, bands 1–5 and 7) were also identified: i.e. thrombospondin 1 (Fig. 9A, band 1), complement factor H–related protein C (Fig. 9A, band 2), coagulation factor XIII A chain (Fig. 9A, band 3), serotransferrin (Fig. 9A, band 4), antithrombin (Fig. 9A, band 5), and metalloproteinase inhibitor 3 (Fig. 9A, band 7). Immunoblot analysis confirmed that GA-BSA could bind to apoE (Fig. 9B). The solid-phase binding and pulldown assays showed that GA-BSA and GA-PA were almost equally recognized by the three apoE isoforms (Figs. 9, C and D). Based on binding of apoE to GA-modified proteins, we examined the changes in antibody response to GA-modified proteins in sera from male control and apoE-deficient hyperlipidemia mice. As shown in Fig. 9E, there was an increased tendency for the serum IgG and IgM titers to GA-BSA and BDA-BSA in apoE-deficient mice compared with control mice. Of interest, the levels of the anti-GA-BSA titers were notably higher than those of the anti-BDA-BSA titers in control and hyperlipidemia mice. Thus, these data suggest that the impaired lipid and lipoprotein metabolism caused by apoE deficiency may accelerate the immune response, leading to overproduction of these antibodies against GA-specific epitopes, including pyrK.

**Immune response to GA-modified proteins**

Given the significant increase in serum levels of antibodies against GA-specific epitopes in apoE-deficient mice, we evaluated the changes in immune response in BALB/c mice after immunization with GA-modified proteins. The mice were immunized every 2 weeks with GA-modified keyhole limpet hemocyanin (GA-KLH), and the IgG and IgM responses to the antigens, including control BSA, GA-BSA, and BDA-BSA (pyrrolated BSA), were examined. As shown in Fig. 10A, mice immunized with GA-KLH displayed significant IgM responses to GA-BSA and pyrrolated BSA. However, only a low IgG response to pyrrolated proteins was observed.

To gain more insight into the immune response to GA-modified proteins, we established three hybridoma clones (GAK1, GAK2, and GAK3) producing mAbs that recognize GA-BSA from GA-KLH–immunized mice (Fig. 10B). GAK1 and GAK2 produced IgG, whereas GAK3 produced IgM. The IgG mAb GAK1 cross-reacted specifically with GA-BSA and glyceraldehyde-modified BSA but did not display detectable binding with pyrrolated proteins. The IgG mAb GAK2 also cross-reacted with the GA-BSA and glyceraldehyde-BSA more prominently.
An endogenous source of lysine N-pyrrolation

than with pyrrolated proteins. However, the IgM mAb GAK3 was rather specific to pyrrolated proteins. In fact, binding of IgM mAb to GA-modified proteins was inhibited by pyrrolated proteins in a dose-dependent manner (Fig. 10C), suggesting that the IgM mAb may have specificity toward an epitope such as pyrK.

Discussion

Endogenous reactive species such as oxidized fatty acids and intermediates of glycolysis mediate covalent modification of proteins under physiological conditions. The ε-aminogroup of lysine is one of the targets of modification and has a major effect on the chemical properties of proteins, leading to important functional and regulatory consequences. Lysine N-pyrrolation, converting lysine residues to pyrK, is associated with an increased negative charge because of neutralization of the lysine residues and with conferring electrochemical properties onto proteins that potentially produce an electrical mimic to DNA (2). Our recent study has revealed that apoE deficiency leads to significant accumulation of pyrrolated serum albumin and is associated with an enhanced immune response (3). This finding strongly suggests that a physiologically relevant molecule might be involved in lysine N-pyrrolation. However, until
now, the endogenous source of pyrrolation was unknown. In this study, based on detection of pyrK in oxidized LDL, we focused our attention on lipid peroxidation and identified the simplest hydroxyaldehyde, GA, as a source of lysine N-pyrrolation. The presence of an endogenous pyrrolylating factor is thought to be an important indication that the processes leading to formation of pyrrolated proteins can take place under physiological conditions.

GA can be enzymatically formed from fructose 1,6-bisphosphate as catalyzed by ketolase in an alternate glycolysis pathway. It is also a common intermediate generated through several nonenzymatic reactions, such as peroxidation of PUFAs (12, 13), sugar fragmentation during glycation (14), myeloperoxidase-catalyzed oxidation of L-serine (15), and oxidative degradation of vitamin C (ascorbic acid) (16). GA produced from activated macrophages and neutrophils has been reported to play an important role in generation of an active ligand for the macrophage scavenger receptor. Because of the presence of multiple sources for GA in vivo, it is still difficult to discern the primary source of GA involved in lysine N-pyrrolation in complex biological matrices. However, identification of GA as a physiological pyrrolylating factor may emphasize the significance of GA metabolism in biological systems and may provide additional insight into the etiologic role of this glycolytic product in the pathogenesis of chronic inflammatory diseases.

GA has facile reactivity with biological materials, including proteins, and has been implicated in the pathogenesis of vascular diseases such as atherosclerosis and renal disorders (14, 17). Upon reaction with proteins, GA primarily reacts with lysine amino groups to form a variety of products, including brown-colored cross-linked structures (18), N\(^\text{N}\)-carboxymethyl)lysine (19), and a pyridinium-containing lysine adduct (17). This study establishes, for the first time, that GA is capable of converting lysine amino groups to pyrK. To the best of our knowledge, few, if any, studies have unequivocally demonstrated formation of pyrK by endogenous molecules. Because the pathway was expected to require an oxidation process, we hypothesized that lysine N-pyrrolation by GA might be explained by a mechanism involving formation of an oxidized intermediate and revealed that oxidative conversion of GA to glyoxal could be involved in formation of pyrK. We propose that formation of pyrK might proceed through reaction mechanisms involving GA and its oxidized product, glyoxal (Fig. 8). First, GA undergoes nucleophilic addition of the lysine amino group to form a GA–lysine Schiff base adduct as the first intermediate, which is converted to a formylmethyl derivative through an Amadori

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**Figure 9. GA-modified proteins act as a ligand of apoE.** A, pulldown assay for detection of binding proteins for GA-modified proteins in mouse serum. BSA coupled to Dynabeads was incubated with 25 mM GA in PBS for 1 or 7 days to obtain GA-modified protein-coupled beads. The mouse serum was incubated with the ligand-coupled beads for 1 h at room temperature. The ligands used were BSA and GA-BSA. Proteins bound to the beads were eluted by addition of sample buffer, heating (95 °C, 5 min), and separation by SDS-PAGE. The proteins indicated by the arrowheads represent thrombospondin 1 (band 1), complement factor H–related protein C (band 2), coagulation factor XIII A chain (band 3), serotransferrin (band 4), antithrombin (band 5), apolipoprotein E (band 6), and metalloprotease inhibitor 3 (band 7), as identified by LC-MS/MS analysis. B, binding of modified proteins to apoE. Serum proteins bound to the beads were prepared and separated by SDS-PAGE as described in A. ApoE was detected by immunoblotting with anti-apoE mAb E6D7 (Abcam). WB, Western blot. C, binding of GA-modified proteins or pentaamines to the apoE isoforms. The apoE isoforms (2 μg/ml) were immobilized on a plate and incubated with biotin-labeled BSA or GA-BSA (50 μg/ml) (left panel) or with biotin-labeled N-pentaamine (PA) or GA-PA (0.5 mM) (right panel) at 37 °C for 1 h. Data are from single experiments performed in triplicate wells and are representative of three individual experiments. The results shown are means ± S.D. (n = 3). D, pulldown assay for binding of GA-modified proteins to the apoE isoforms. Three apoE isoforms were incubated separately with ligand-coupled beads for 1 h at room temperature. The ligands used were BSA, BDA-BSA, and GA-BSA. Proteins bound to the beads were eluted by addition of sample buffer, heating, and separation by SDS-PAGE. The apoE isoforms were detected by immunoblotting with anti-apoE mAb E6D7. E, levels of the antibody titers against antigens (BSA, BDA-BSA, and GA-BSA) in sera from 12 weeks of male control and spontaneously hyperlipidemic mice. Elevations of IgG (left panel) or IgM (right panel) immune responses in the serum samples were measured by ELISA using native BSA, BDA-BSA, and GA-BSA as the coating antigens. *, p < 0.05; **, p < 0.01. The results shown are means ± S.D. (n = 3).
rearrangement reaction. The Amadori product then reacts with glyoxal via a nucleophilic addition reaction to generate a tertiary amine derivative that can be converted to pyrK through aldol condensation followed by dehydration reactions. Because GA and glyoxal are also formed during glycation, they may be a common intermediate in formation of pyrK during oxidation of carbohydrates and lipids. Thus, glycation as well as lipid peroxidation may be important sources of pyrK in tissue proteins in vivo.

Various lipid peroxidation-derived aldehydes have been identified as sources of pyrrole derivatives upon reaction with primary amines (4–10). Evidence of the biological occurrence of these pyrrole derivatives bound to proteins has been provided in a variety of diseases, including atherosclerosis (20, 21). In contrast to other lysine adducts, pyrrole-containing adducts are unique because of the presence of an amino charge-neutralized pyrrole structure. Pyrrolation of proteins by 1,4-butanedial indeed confers electronegativity and electronic properties on proteins. The pyrrole ring, a ubiquitous structural motif found in a wide range of biologically active natural products and pharmaceutically active agents, is planar and electron-rich and highly susceptible to electrophilic attack and oxidation. It is also involved in π–π stacking and hydrogen-bonding interactions and displays a variety of pharmacological properties. Thus, formation of a pyrrole structure is likely to have a dramatic effect on the structure and function of proteins. Moreover, the results of this study, in combination with published evidence of formation of GA-modified proteins in vivo (17), point to the possibility that protein-bound pyroles could play roles in inflammatory diseases such as atherosclerosis. However, the potential role of protein-bound pyroles in physiology and pathophysiology needs to be explored further.

It has been postulated that proteins covalently modified with products of diverse classes of oxidative reactions are damage-associated molecular patterns (DAMPs). DAMPs can be ligands of multiple proteins and bind to pattern recognition receptors (PRRs) such as Toll-like receptors, which, in turn, may lead to innate and adaptive immune responses. They also mediate homeostatic functions following inflammation and cell death. It has also been shown that DAMPs, possessing an
exposed epitope, are recognized by soluble PRRs such as innate antibodies (22, 23). We recently established apoE as a PRR for pyrK-containing proteins. Because apoE serves as a bridging molecule for cellular binding of pyrrolated proteins (3), the protein is likely to play a role in providing homeostatic responses to pyrK-containing proteins, including GA-modified proteins, ubiquitously generated in biological systems. Strikingly, deficiency of apoE is associated with a significant increase in IgG and IgM titers against GA-derived epitopes. apoE-deficient mice exhibit evidence of markedly increased covalent modification of lysine residues in proteins, as assessed by formation of a major lipid peroxidation–derived lysine adduct, N^ε-(8-carboxyoctanyl)lysine, and autoantibodies to oxidized lipoproteins (3, 24). The most likely scenario is that deficiency of apoE accelerates peroxidation of fatty acids, which may be associated with accumulation of GA and GA-modified proteins, leading to overproduction of autoantibodies to pyrrolated proteins. However, the causal relationship between lysine N-pyrrolation and an enhanced autoimmune response to GA-specific epitopes, including pyrK, remains unclear.

In our previous studies, we showed that immunization of BALB/c mice with pyrrolated proteins accelerated production of autoantibodies (2). In addition, age-dependent increases in IgG and IgM titers to dsDNA and pyrrolated proteins were observed in systemic lupus erythematosus-prone MRL-lpr mice (2) and in apoE-deficient mice (3). In this study, to evaluate the biological significance of GA as a source of lysine N-pyrrolation, we examined the antigenic property of GA-modified proteins and observed that immunization with GA-modified BSA induced robust IgG production to pyrrolated proteins (Fig. 10), suggesting that GA-specific epitopes could induce the typical B cell memory. A significant increase in the IgM titer to pyrrolated proteins was also observed. The data suggest that the epitopes could also act on the innate immune response. Based on the enhanced immune response to pyrrolation by GA-modified proteins, we isolated three hybridoma clones (GAK1, GAK2, and GAK3) producing mAbs specific to GA-modified proteins from mice and found that the mAb GAK3 recognized pyrrolated proteins as well as GA-modified proteins. More interestingly, mAbs GAK1 and GAK2 were IgG, whereas mAb GAK3, cross-reacting with pyrrolated proteins (BDA-BSA), was IgM. The mechanism for elevation of IgM titers and enhanced production of IgM by immunization with GA-modified proteins is presently unknown. However, natural IgM antibodies are products of the B cell population selected by an antigen. Thus, it is likely that GA-modified proteins containing pyrK could be involved in expansion of the established B-1 cell clones, leading to production of IgM.

In summary, we searched for an endogenous pyrrolating agent and identified GA as a source of pyrK. We also performed mechanistic studies of lysine N-pyrrolation by GA and suggest that reactions might include oxidation of GA, generating glyoxal, followed by condensation reactions of the lysine amino groups with GA and glyoxal. To gain insight into the functional significance of GA-mediated lysine N-pyrrolation in proteins, we characterized GA-modified proteins as ligands of apoE, a binding target of pyrK and pyrrolated proteins, and revealed that GA-modified proteins could be antigens to generate anti-pyrrole antibodies. These findings uncover a novel function of GA as an endogenous source of DNA-mimicking pyrrolated proteins and suggest that GA-derived pyrK residues generated in proteins may play a role in innate and autoimmune responses associated with glucose metabolism and oxidative stress.

### Experimental procedures

#### Materials

BSA and p-aminobenzoic acid (ABA) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). SYBR Green I was obtained from Invitrogen. PUFAs were obtained from Cayman Chemical. 4-Aminobenzoic acid-2,3,5,6-d₄ acid was obtained from CDN Isotopes. Glycolaldehyde was obtained from Sigma. The recombinant human apoE isoforms (E2, E3, and E4) were obtained from PeproTech. SYBR Green I, Dynabeads M-270 carboxylic acid, and EZ-link Sulfo-NHS-LC-biotin were obtained from Invitrogen. The IgM mAb ADL19 was established from MRL-lpr mice (25). All other reagents used in this study were of analytical grade and obtained from commercial sources.

#### LC-ESI-MS/MS

The mass spectrometric analyses were performed using the Acquity TQD system (Waters) equipped with an ESI probe and interfaced with a ultra-performance liquid chromatography system (Waters).

#### In vitro oxidation of LDL

LDL (1.006–1.063 g/ml) was prepared from plasma of healthy humans by sequential ultracentrifugation and then extensively dialyzed against PBS (10 mM sodium phosphate buffer (pH 7.4)) containing 0.3 mM EDTA at 4 °C. The LDL used for oxidative modification by Cu^{2+} was dialyzed against a 1000-fold volume of PBS at 4 °C. The oxidized LDL was prepared as described previously (26). Cu^{2+} oxidation of LDL was prepared by incubating 0.5 mg/ml of LDL with CuSO₄ (5 μM) in PBS at 37 °C. After incubation, the reaction mixture was dialyzed against a 1,000-fold volume of PBS.

#### In vitro modification of BSA

Modification of BSA by lipid peroxidation products was performed by incubating BSA (1.0 mg/ml) with 12.5 mM PUFAs in the presence of 200 μM Cu^{2+} and 10 mM H₂O₂ in 200 μl of 50 mM sodium phosphate buffer (pH 7.2) in atmospheric oxygen at 37 °C. After 0.5, 1, 3, 6, 9, and 24 h, aliquots were collected, and the reaction was terminated by adding EDTA (25 mM). Modification of BSA by carbohydrates and their related metabolites was performed by incubating BSA (1.0 mg/ml) with 10 mM carbohydrates and their related metabolites in PBS at 37 °C for 24 h.

#### Preparation of GA-modified pentylamine biotin

GA-modified pentylamine biotin was prepared upon incubation of 10 mM of EZ-link® pentylamine biotin with 10 mM GA in PBS at 37 °C for 24 h.
An endogenous source of lysine N-pyrrolation

Staining of proteins by DNA intercalator

Electrophoresis was performed in a non-denaturing polyacrylamide gel (9% acrylamide) in 2× TAE buffer (80 mM Tris acetate and 2 mM EDTA (pH 8.0)). Tris-glycine buffer (25 mM Tris and 192 mM glycine (pH 8.4)) was used as the running buffer. 10 μl of 1 mg/ml protein was mixed with 2 μl of 6× loading buffer (0.03% bromphenol blue, 10 mM Tris-HCl (pH 7.6), 0.6 mM EDTA, and 60% glycerol) and electrophoresed in the gel at 100 V. After electrophoresis, the gels were rinsed in ultrapure water and stained with 2.5 mg/ml SG dissolved in TAE buffer for 30 min. Fluorescence scanning of the gels was carried out using an ImageQuant LAS 4000 system (GE Healthcare and Life Sciences). After SG staining, the gels were fixed and stained with Coomassie Brilliant Blue.

For measurement of SG-stainable proteins in a microplate, a 100-μl aliquot of the proteins (0.1 mg/ml) in PBS was added to each well of a 96-well microtiter plate. Immediately after adding 100 μl of a 5 × 10−3 dilution of SG solution, the fluorescence (excitation/emission at 494/521 nm) was read using a microELISA plate reader.

DNPH derivatization of aldehydes

HPLC-fractionated aqueous phases of oxidized EPA or DHA were evaporated and dissolved in 100 μl of H2O. Samples were derivatized with an equal volume of 0.1% DNPH in 2 N HCl at room temperature for 30 min. The DNPH derivatives were extracted with chloroform, evaporated, and dissolved in 200 μl of ethanol. 20 μl of the aliquot was injected and analyzed by HPLC on a Sunnigest RP-AQUA column (4.6 mm inner diameter × 250 mm; ChromaNik, Osaka, Japan) eluted with a gradient of water containing 0.1% TFA (solvent A) with acetonitrile containing 0.1% TFA (solvent B) (time 0–5 min, 0% B; 5–30 min linear increase of B up to 100%; 30–35 min, 100% B) at a flow rate of 4 ml/min. The elution profiles were monitored by absorbance at 220 nm. The eluate was collected at 1-min intervals. The collected products were evaporated and incubated with 1 mg/ml BSA at 37 °C for 4 days, and production of SG-stainable proteins was determined in a microplate.

Determination of glycolaldehyde

To prepare the internal standard, 20 mM 4-aminobenzoic acid (4-AB) was treated with 10 mM GA and 25 mM NaCNBH3 at 37 °C for 2 h in the dark. Isotope-labeled GA-ABA was purified by HPLC, evaporated, and dissolved in water. The concentration of the products was measured by absorbance at 280 nm using ABA as a standard. For GA determination, 15 μl of samples was incubated with 25 mM ABA and 25 mM NaCNBH3 in 100 μl of 0.5 mM diethylenetriamine-N,N,N′,N′-pentaacetic acid at 37 °C for 2 h in the dark. The internal standard, [2H4]GA-ABA, was added to the samples prior to incubation. After the reaction, methanol (300 μl), chloroform (100 μl), and water (300 μl) were added and vortexed. After centrifugation, the upper phase was collected, evaporated, and then reconstituted in 100 μl of H2O:MeOH (1:1). The AB derivative of GA was measured using a stable isotope dilution-based LC-ESI-MS/MS technique. The column and gradient conditions were the same as those for measurement of the DNPH derivatives. A mass spectrometry analysis in positive ion mode was performed in multiple reaction monitoring (MRM) mode (cone potential, 30 eV; collision energy, 15 eV). MRM transitions monitored were as follows: [2H4]GA-ABA, m/z 186.4 > 124.3; GA-ABA, m/z 182.4 > 120.3. The amount of the GA-ABA was quantified by the ratio of the peak area of GA-ABA and of the GA-ABA stable isotope.

Analysis of pyrK and pyrNAK

To identify a lipid peroxidation-derived source of pyrK, the aqueous phase of the oxidized PUFAs (EPA and DHA) was subjected to reverse-phase HPLC on a Sunnigest RP-AQUA column (10 mm inner diameter × 250 mm, ChromaNik) eluted with a gradient of water containing 0.1% TFA (solvent A) with acetonitrile containing 0.1% TFA (solvent B) (time 0–5 min, 0% B; 5–30 min linear increase of B up to 100%; 30–35 min, 100% B) at a flow rate of 4 ml/min. The elution profiles were monitored by absorbance at 220 nm. The eluate was collected at 1-min intervals. The collected products were evaporated and incubated with 1 mg/ml BSA at 37 °C for 4 days, and production of SG-stainable proteins was determined in a microplate.

Lipid peroxidation was performed by incubating 12.5 mM PUFA ethyl esters (EPA and DHA) in the presence of 200 μM Cu2+ and 10 mM H2O2 in 200 μl of 50 mM phosphate buffer (pH 7.2) in atmospheric oxygen at 37 °C. After 24 h, the reaction mixture was extracted with an equal volume of chloroform: methanol (2:1) and vortexed. After centrifugation, the upper (aqueous) and lower (organic) phases were collected and incubated with BSA at 37 °C for 48 h. Formation of pyrrolated proteins was examined by nondenaturing PAGE followed by staining with SG.

Identification of a lipid peroxidation-derived source of pyrK

The protein samples were suspended in H2O (400 μl), and 400 μl of chloroform:methanol (1:3 (v/v)) was added. The tubes were agitated on a vortex mixer and centrifuged at 5000 rpm for 10 min. An interphase was formed, and the fluid above was removed. The remaining solution was evaporated. The proteins were suspended in H2O (50 μl) and mixed with 300 μl of 4 N NaOH and 10 μl of 10 μM [U-13C6,15N2]-pyrK and then hydrolyzed by heating for 15 h at 110 °C. The resulting solution was neutralized with hydrochloric acid. After digestion, the samples were partially separated using Oasis hydrophilic–lipophilic balance cartridges (Waters). After sample loading, the hydrophilic–lipophilic balance cartridges were washed with 2 ml of H2O, and N+-pyrK was eluted with 1 ml of 20% acetonitrile. The samples were then dried, dissolved in 100 μl of H2O, and subjected to LC-ESI-MS/MS analysis as reported previously (2).
N-\textsuperscript{\textgamma}-pyrrolylation of NAK by GA was performed by incubating 1 mM NAK with 10 mM GA in PBS at 37 °C for 18 h. pyrrolylation was measured by LC-ESI-MS/MS in MRM mode (positive ion mode; cone potential, 25 eV; collision energy, 20 eV). The MRM transition monitored was 239.3 to 150.1. Sample injection volume of 5 μl each was separated on a Waters BEH C18 1.7-μm microM column (100 × 2.1 mm) at a flow rate of 0.3 ml/min. A discontinuous gradient was used by solvent A (H₂O containing 0.1% formic acid) with solvent B (acetonitrile containing 0.1% formic acid) as follows: 5% B at 0 min, 30% B at 8 min, and 99% B at 9 min.

**ELISA**

The antibody titer against the modified proteins in the sera or monoclonal antibodies was analyzed by direct antigen ELISAs. A 100-μl aliquot of the antigen solution (50 μg/ml) was added to each well of a 96-well microtiter plate and incubated for 20 h at 4 °C. The antigen solution was then removed, and the plate was washed with PBS containing 0.5% Tween 20 (PBS/Tween) and a plate washer (Auto Mini Washer AMW-8, Biotec). Each well was incubated with 200 μl of 4% Blockace (Yukijirushi, Sapporo, Japan) in PBS/Tween for 60 min at 37 °C to block the unsaturated plastic surface. The plate was then washed three times with PBS/Tween. A 100-μl aliquot of a 1000-fold (for IgG) or 2500-fold (for IgM) dilution of serum was added to each well and incubated for 2 h at 37 °C. After discarding the supernatants and washing three times with PBS/Tween, 100 μl of a 5 × 10\textsuperscript{3} dilution of goat anti-mouse IgG or IgM conjugated to hors eradish peroxidase in PBS/Tween was added. After incubation for 1 h at 37 °C, the supernatant was discarded, and the plates were washed three times with PBS/Tween. The enzyme-linked antibody bound to the well was revealed by adding 100 μl/well of 1,2-phenylenediamine (0.5 mg/ml) in 0.1 M citrate/phosphate buffer (pH 5.5) containing 0.003% hydrogen peroxide. The reaction was terminated by addition of 2 M sulfuric acid (50 μl/well), and the absorbance at 492 nm was read using a micro-ELISA plate reader.

**Competition assays** were performed by ELISA, in which binding of the IgM mAb to the coated GA-BSA was in competition with pyrrolylated proteins (BDA-BSA). The antibody was preincubated with the indicated concentrations of the competitors (BSA or BDA-BSA) before addition to the GA-BSA-coated ELISA plate. The bound biotin-labeled protein was detected with NeutrAvidin coupled to HRP. The substrate was measured as already described.

**Pulldown assay**

BSA coupled to the Dynabeads was incubated with 25 mM GA in PBS for 1 or 7 days to obtain GA-modified protein-coupled beads. The BDA-BSA beads were prepared by incubating the BSA coupled to the beads with 1 mM BDA in PBS for 24 h. The beads (2 × 10\textsuperscript{7}) were then added to microcentrifuge tubes and incubated with 500 μl of 10× dilution mouse serum in PBS/Tween for 3 h at room temperature. After washing three times with PBS/Tween, the binding proteins were eluted by adding sample buffer and heating (95 °C, 5 min). Protein separation by SDS-PAGE performed under reducing conditions was processed for tryptic digestion and LC-MS/MS analysis (LC and auto sampler: Zaplous Advance nano UHPLC HTS-PAL system, AMR; MS: Orbitrap VELOS ETD, Thermo Fisher Scientific). Peptides were loaded onto a trap column (0.3 mm inner diameter × 5 mm, 5 μm, L-column, CERI) and directly connected to a Zaplous α Pep-C18 packed column (3 μm, 0.1 × 150 mm, AMR). Mobile phase A was 0.1% formic acid in ultrapure water (Wako Chemicals), and mobile phase B was 100% acetonitrile. The gradient was 5% B to 45% B from 0 – 60 min at a flow rate of 500 nl/min. The mass spectrometer was operated in positive ionization mode, and isolated charged ions were fragmented by collision-induced dissociation in a linear ion trap mass spectrometer. Proteins were identified with Proteome Discoverer 2.1 search algorithms using the *Mus musculus* database from Uniprot. The MS parameters are listed in Table S8.

**Immunoblot analysis**

The samples were run on 10% SDS-polyacrylamide gels, and the gels were transblotted onto a PVDF membrane (GE Healthcare), incubated with Blocking One (Nacalai Tesque) for blocking, washed, and then incubated with an anti-apoE antibody (F-9, Santa Cruz Biotechnology) in Tris-buffered saline with 0.1% Tween-20 (TTBS) overnight at 4 °C. After washing, the membrane was incubated with 2000× HRP-linked secondary antibody for 1 h at room temperature. This procedure was followed by addition of ECL reagents. The bands were visualized using a Cool Saver AE-6955 (ATTO, Tokyo, Japan).

**Animals**

All experiments were performed according to the guidelines of the Animal Usage Committee of the Faculty of Agriculture, University of Tokyo (Tokyo, Japan) and approved by the committee (permission no. P19-047). Male BALB/c and male apoE-deficient mice (C.KOR/StmSlc-Apo\textsuperscript{eo}) were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in a temperature-controlled pathogen-free room with light from 7:00 to 19:00 (daytime) and had free access to standard food and water. Blood was collected from the tail vein and allowed to stand for 2 h at room temperature, and then the sera were collected by centrifugation at 3500 rpm for 10 min and stored at −80 °C until used.

**Preparation of mAbs**

mAbs were prepared from BALB/c mice immunized with GA-modified KLH. Immunogen was prepared by incubating KLH (1.0 mg/ml) with 25 mM GA in PBS (pH 7.4) at 37 °C for 7 days. We immunized female mice in week 6 with complete Freund adjuvant and 0.5 mg of immunogen and boosted in weeks 8, 10, and 12 with incomplete Freund adjuvant by emulsifying and intraperitoneal injection. On week 13, spleen cells from immunized mice were fused with P3/U1 murine myeloma cells and cultured in hypoxan-
An endogenous source of lysine N-pyrrolation

thine/aminopterin/thymidine selection medium. The culture supernatants of the hybridoma were screened using an ELISA with GA-BSA as the antigen. Hybridoma cells corresponding to supernatants that were positive on GA-BSA and negative on native BSA were then cloned by limited dilution. After repeated screenings, three clones showing specific recognition of GA-BSA were obtained.

Statistical analysis

The data represent the mean ± S.D. where indicated. Statistical significance was evaluated using unpaired Student’s t test or, when appropriate, Dunnett’s test.

Data availability

The mass spectrometry raw data have been deposited to ProteomeXchange (PXD018423).

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Data availability

The mass spectrometry raw data have been deposited to ProteomeXchange (PXD018423).

References


