Pro-inflammatory Effects of Advanced Lipoxidation End Products in Monocytes.

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Running title: MDA-Lys Induced Cytokine Expression

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ABSTRACT

Background: The reactions of carbohydrate or lipid derived intermediates with proteins lead to the formation of Maillard reaction products, which subsequently lead to the formation of advanced glycation/lipoxidation end products (AGE/ALEs). Levels of AGE/ALEs are increased in diseases like diabetes. Unlike AGEs, very little is known about ALE effects in vitro. We hypothesized that ALEs can have pro-inflammatory effects in monocytes.

Methods and Results: Synthetic ALE (malondialdehyde-lysine, MDA-Lys) (50µmol/l) could induce oxidant stress and also activate the transcriptional factor NF-κB in THP-1 monocytes. MDA-Lys also significantly increased the expression of key candidate pro-inflammatory genes, Interferon-γ-inducible protein-10, β1 and β2-Integrins, cyclooxygenase-2, monocyte chemoattractant protein-1 (MCP-1), interleukins-6 and-8, and iNOS that are also associated with monocyte dysfunction. In a profiling approach, conditioned media from THP-1 cells cultured either in normal glucose (NG, 5.5 mM) or treated with MDA-Lys or MDA alone were hybridized to arrays containing antibodies to 120 known human cytokines/chemokines. Several key target pro-inflammatory proteins were significantly induced by MDA-Lys relative to NG or MDA alone, including MCP-1, TNF ligand superfamily member-14, Chemokine CC motif Ligand-11 (CCL-11), Growth Related Oncogene α, β, γ, and Chemokine CXC motif Ligand-13. Pathway analyses with bioinformatics software identified a network of chemokine signaling amongst MDA-Lys regulated genes. MDA-Lys also increased monocyte binding to vascular smooth muscle and endothelial cells. Furthermore, plasma from diabetic rats showed significantly higher levels of MDA-Lys as well as CCL11.

Conclusion: These new results suggest that ALEs can promote monocyte activation and vascular complications via induction of inflammatory pathways and networks.
Maillard reaction products are formed by reactions of proteins with carbohydrate or lipid derived intermediates and reactive carbonyls, such as malondialdehyde (MDA), and this subsequently leads to the formation of advanced glycation/oxidation end products (AGE/ALEs). AGE/ALE levels increase during aging and in diseases like diabetes (1, 2). MDA, a naturally occurring dialdehyde produced in cell membranes by lipid peroxidation, is a strong alkylating agent of primary amino groups in proteins and free amino acids. It occurs mainly in the form of an adduct with lysine, indicating that its predominant reaction in vivo is with lysine residues of proteins (1-3). The biological functions of MDA-lysine [N (epsilon)-(2-propenal) lysine] adducts generated with proteins have not been well studied. MDA-lysine (MDA-Lys) epitopes are however closely associated with atherosclerosis (1, 2, 4) and recognized as markers of oxidation. Oxidative stress is implicated in the pathogenesis of numerous diseases including diabetes mellitus and atherosclerosis. It promotes the formation of oxidized low-density lipoprotein (ox-LDL), which has pleiotrophic effects in cells involved in atherosclerosis (5). Polyunsaturated fatty acid components of LDL are susceptible to peroxidation by reactive oxygen species (ROS), leading to the formation of major end-products of toxic and highly reactive aldehydes such as MDA and 4-hydroxynonenal (HNE) that can attack intracellular and extracellular proteins (1, 2, 6). These end-products are frequently measured as indicators of lipid peroxidation and oxidative stress in vivo. They form Schiff-base adducts with lysine residues and cross-links with proteins in vitro and in vivo. HNE also reacts with lysines, primarily via Michael addition reaction (1-4). These lipid-protein adducts are termed ALEs. Such lipid-aldehyde conjugation of proteins can have diverse effects on cell function, including cross-linking of cell surface receptors, alteration of enzyme activities and modification of lipoproteins. However, very little is known about their mechanisms of action.

Extensive accumulation of MDA-lysine and carboxymethyl-lysine (CML) was shown in diabetic kidneys (7). Anti MDA-lysine adduct antibody studies demonstrated extensive presence of MDA-modified proteins in atherosclerotic lesions, primarily in macrophage-derived foam cells (1, 6, 8, 9). ALEs such as MDA- and HNE-protein adducts, and AGEs such as CML, accumulate during the development of diabetes and are believed to mediate diabetic complications (1, 6, 7, 10, 11).

Certain key factors, including oxidant stress, increased flux through the polyol and hexosamine pathways, protein kinase C (PKC) and mitogen activated protein kinase (MAPK) activation, and inflammatory gene expression (12-14), have been implicated in diabetes and diabetes induced accelerated atherosclerosis. Several of these pathways can be triggered by AGEs (11, 15, 16). In monocytes, these pathways can lead to cellular dysfunction, adhesion to the endothelium and transmigration into the subendothelial space, all key events in the pathogenesis of atherosclerosis. Several monocyte-macrophage inflammatory cytokines and chemokines have been implicated in these processes.

We and others recently demonstrated that AGEs and ligands of the receptor for AGE, such as S100b, can induce potent inflammatory cytokines and chemokines in human THP-1 monocytes as well as peripheral blood monocytes, and that these genes were also augmented in monocytes from diabetic patients (14, 17-21). However, it is not known whether ALEs can elicit similar effects in monocytes or other target
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cells affected by diabetes. We therefore examined the cellular actions of ALEs by evaluating the effects of MDA-Lys, a prototype ALE, in monocytes. We observed that MDA-Lys induces not only known inflammatory genes but also novel ones such as eotaxin, and also promotes monocyte activation and dysfunction. We identified NF-κB as a key MDA-Lys activated transcription factor. Furthermore, MDA-Lys seemed to act partly via the AGE receptor, RAGE. In addition, we observed increased levels of MDA-Lys and eotaxin in the plasma of diabetic rats, demonstrating in vivo relevance.

MATERIALS AND METHODS
(Other Methods available in the Online Supplement [available at http://diabetes.diabetesjournals.org])

Materials. Inhibitors used: SB202190, GFX, and N-acetylcysteine (NAC), were purchased from Calbiochem (San Diego, CA); PD98059 was from Cell Signaling (Beverly, MA). Human Cytokine antibody Arrays and corresponding reagents were from Ray Biotech (Norcross, GA). 32P-γ-ATP (3000 Ci/mmol) was from New England Nuclear (Boston, MA). RT-PCR and qPCR reagents were from Applied Biosystems (Foster City, CA) and Quantum RNA 18S Internal Standards from Ambion Inc. (Austin, TX). Effectene transfection reagents, plasmid DNA isolation kits, RNeasy, Oligotex kits (for mRNA preparation) and real time PCR primers for CCL11, 18 and TNFSF14 were from Qiagen, Inc. (Valencia, CA). ELISA kit for CCL11 was from R&D system (Minneapolis, MN). Luciferase assay system was from Promega, Inc. (Madison, WI). MDA-Lys was quantified with a kit (BIOXYTECH-MDA-586) from OxisResearch (Foster city, CA). Oligonucleotides containing NF-kB, AP-1 and Egr-1 consensus sequences were from Santa Cruz Biotechnology.

Cell Culture and Treatments. Human THP-1 monocytic cells were cultured in RPMI-1640 medium with either 5.5 mM D-glucose (normal glucose, NG) or 50μm/l (10μg/ml) MDA-Lys at 37°C. In some experiments, THP-1 cells were pre-treated with SB202190 (p38MAPK inhibitor, 1 μmol/l), or bis-indolylmaleimide (GFX, PKC inhibitor, 5 μmol/l), or PD98059 (ERK MAPK pathway inhibitor, 25 μmol/l), or NAC (antioxidant, 100 μmol/l). The cells were then incubated in NG or MDA-Lys media for a further 8-12h. Human Vascular smooth muscle cells (HVSMCs) purchased from Clonetics (San Diego, CA) and Human Umbilical Vein Endothelial Cells (HUVEC) from Cambrex (East Rutherford, NJ) were cultured according to manufacturer's specifications.

Biological network analysis. The data list of genes upregulated by MDA-Lys were analyzed and networks generated by Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Details are provided in the Results, Legends and Online Supplement.

Measurement of secreted CCL11 levels. CCL11 was assayed in conditioned media from THP-1 cells (5X10^5 cells/ml) cultured in NG, MDA-Lys or MDA alone for 8h, using a specific ELISA kit.

MDA-Lys Measurement. Rat plasma MDA-Lysine levels were measured using MDA-586 assay kit according to manufacturer's instructions.

Data Analyses. Data are expressed as mean ± SEM of multiple experiments. Paired Student’s t-tests were used to compare two groups and ANOVA with Dunnett’s post tests for multiple comparisons.

RESULTS
MDA-Lys induces Oxidant Stress in THP-1 cells. We first examined whether MDA-Lys treatment can induce oxidant stress in monocytes similar to AGEs. We chemically synthesized MDA-Lys needed for these
studies and verified its purity by MS/MS analysis (Supplement Methods and Fig. S1). Purified monomer was used in all experiments. THP-1 monocytes were treated with MDA-Lys for 30 min and stained with cell-permeable fluorescent probes 2′-7′-dichlorofluorescein (DCF) diacetate to detect oxidant stress, or dihydroethidine (DHE) to detect superoxide formation, using a fluorescent microscope. MDA-Lys induced a significant increase in oxidant stress (Fig.1A, upper panel and Fig. 1B, bar graph) and also superoxide formation (Fig. 1A lower panel and Fig.1B, bar graph) compared with untreated control cells grown in NG.

**MDA-Lys induces NF-κB activation.** We next examined whether MDA-Lys can activate oxidant sensitive transcription factors such as NF-κB, AP-1 and Egr-1, which are known regulators of pro-inflammatory genes. Electrophoretic mobility shift assays (EMSAs) were first performed in THP-1 cells after 1h incubation with MDA-Lys. Fig.1C shows that MDA-Lys can increase DNA binding activities for NF-κB (arrow) (lanes 2 and 3 vs control lane 1), a slight increase in AP-1 (lane-7 vs 6), but not Egr-1 (lane 9 vs 8). To confirm whether the NF-κB complex contains the transcriptionally active p65 subunit, we performed additional EMSAs after pre-treatment with an anti-p65 antibody and observed a super shifted band in antibody treated samples (lanes 4 and 5). To further confirm whether increased DNA binding was associated with transcriptional activity, THP-1 cells were next transiently transfected with plasmids containing heterologous promoters driven by multiple NF-κB or AP-1 response elements upstream of luciferase reporter. MDA-Lys significantly increased NF-κB promoter-driven luciferase activity (Fig. 1D) but not AP-1 (not shown). Furthermore Western blots showed an increase in phosphorylation of NF-κB p65 by MDA-Lys (Fig.1E) further supporting increased NF-κB activation.

We next examined whether MDA-Lys can transactivate the promoters of key inflammatory genes known to be regulated by NF-κB and by AGEs in monocytes, namely MCP-1, IP-10 and COX-2 (14, 18-20). THP-1 cells were transiently transfected with plasmids containing luciferase gene under control of human IP-10 (-438/+97), MCP-1 (-3011/+52) or COX-2 (-860/+127) promoter fragments. MDA-Lys significantly increased promoter activities of COX-2 (Fig.1G) and MCP-1(Fig.1H), but not IP-10 (Fig.1F). To further confirm the role of NF-κB, we co-transfected cells with an NF-κB super-suppressor IkB-α (mutant) plasmid along with the promoter constructs. Fig-1G shows MDA-Lys induced COX-2 promoter activations was completely blocked by this mutant while MCP-1 promoter activation was partially, but significantly blocked (Fig-1H). These results suggest that MDA-Lys can induce COX-2 and MCP-1 transcription via NF-κB activation, while that of IP-10 may be due to mRNA stabilization as noted for IP-10 induction by the RAGE ligand, S100b (20), or that the MDA-Lys-response elements for IP-10 are further downstream of the IP-10 promoter fragment used.

**MDA-Lys induces the expression of key atherogenic genes associated with monocyte activation.** Initial RT-PCR analysis of THP-1 cells treated with increasing concentrations of MDA-Lys (1,5, 10 and 20µg/ml), showed that maximal induction of COX-2 mRNA occurred with 10µg/ml (50µmol/l) (data not shown) and hence this dose of MDA-Lys was used in subsequent experiments. Evidence shows that AGEs and ligands of RAGE can increase the expression of several inflammatory cytokines and chemokines in monocytes such as β-integrins, RAGE, IL-1β, COX-2, iNOS, COX-1, IP-10 and MCP-1 (14, 18-20). In a candidate gene approach, we therefore tested whether MDA-Lys can regulate these genes in THP-1 cells by performing multiplex RT-PCRs using specific
primers for the human genes (Supplement Table S1) paired with 18S rRNA primers as internal controls. Results showed that MDA-Lys increased, in a time-dependent fashion (1-24 hr), the expressions of MCP-1, β1 and β2-integrins, RAGE, IP-10, CCR2, iNOS, IL-6, IL-8 and COX-2, but not β6-integrin, IL-1β, CD-36 (Fig.2A). Bar graph quantification in Fig-2B obtained from multiple independent experiments at 4, 8 or 24hr shows that the stimulatory effects on these molecules are significant.

**MDA-Lysine induces proinflammatory cytokines and chemokines.** The candidate gene approach demonstrated that MDA-Lys and potentially ALEs can induce key known pro-inflammatory and monocyte activating genes that have also been shown to be induced by high glucose, AGEs and RAGE ligands in monocytes (14, 18-20). However, since ALEs may also induce other ALE-specific inflammatory genes in monocytes, we used commercial human cytokine antibody arrays to profile proteins regulated by MDA-Lys. Results from three independent experiments shown in Fig.3 and Supplement Table S2 indicate that the levels of 20 cytokines levels were increased by 1.5 to 5 fold by MDA-Lys relative to control (NG), including CCL11, CCL18, CCL28, TNFSF14, MCP-1 and MCP-2, while 6 cytokines were decreased including CCL1, CXCL13, CSF-2, and MCP-3. These results show for the first time that multiple inflammatory cytokines and chemokines are regulated by ALEs such as MDA-Lys in monocytes. Interestingly, many of these genes (Supplement Table S2, Figs.2 and 3) are reported to be regulated by NF-κB, suggesting an important regulatory role for NF-κB in the effects of ALEs similar to AGEs. However, ALEs appear to also augment key new genes not reported to be induced by AGEs.

We next examined whether some of these key protein targets induced by MDA-Lys are also modulated at the transcriptional level by analyzing mRNA levels of CCL11, CCL18 and TNFSF14. RT-PCR data in Figure 4A show that MDA-Lys clearly increased mRNA levels of TNFSF14 and CCL11, but not CCL18 despite increased CCL18 protein levels (Fig-3 and Supplement Table S2). In addition, we performed real time qPCR of RNA samples from THP-1 cells treated 1-24 hr with MDA-Lys. Bar graphs in Fig-4B show that MDA-Lys significantly increased CCL11 and TNFSF14 mRNA levels with peaks at 8h, but not CCL18 mRNA. These results along with the previous data demonstrate that MDA-Lys can regulate the expression of inflammatory genes in monocytes via both transcriptional and post-transcriptional mechanisms.

In order to verify that MDA-Lys can exert similar effects even in primary monocytes, human peripheral blood monocytes were treated for 8h with MDA-Lys and mRNA expressions analyzed by RT-PCR. Fig-4C shows CCL11 and TNFSF14 mRNA levels were increased, but not CCL18, similar to data obtained in THP-1 cells. We next examined whether these factors are also induced by AGEs. THP-1 cells were treated with or without methyl-glyoxal modified BSA prepared as described (19). This AGE-BSA could also induce TNFSF14 and CCL11 mRNAs but not CCL18 (Fig. 4D) similar to the effects of MDA-Lys (Fig. 4C).

In order to determine whether MDA-Lys can exert some of its cellular effects via the AGE receptor RAGE, THP-1 cells were pretreated with a specific anti-RAGE antibody for 1hr prior to MDA-Lys. This led to almost complete blockade of MDA-Lys induced CCL11 mRNA (Fig.4A lanes 9 and 10, and Fig-4B middle panel) but not TNFSF14 (Fig.4A lanes 7 and 8, and Fig.4B left panel), suggesting that MDA-Lys can signal not only through RAGE but also via other as-yet unidentified receptors. Taken together, these observations demonstrate that
ALEs can enhance the expression of key inflammatory chemokines that promote monocyte adhesion, transmigration and foam cell formation as seen in diabetes and its complications.

*Ingenuity Pathway Analysis of MDA-Lys regulated molecules.* In order to identify potential networks of inflammatory signaling that may be triggered by MDA-Lys, we utilized Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) to uncover diabetes related biological networks amongst MDA-Lys regulated proteins and mRNAs (Supplement Table S2, Fig.2 and Fig.3). Interestingly, this data mining tool uncovered two groups of networks with high scores (Table-1) from the MDA-Lys-induced factors, that include proteins involved in immune response, cellular movement and cell-to-cell signaling. From these, we chose candidates associated with transcription, gene expression, cellular activation/deactivation. The resulting network (Fig.5A) placed our candidate genes in canonical pathways with significant association with chemokine signaling (Fig.5B) (see Legends and Supplement for additional details) suggesting that MDA-Lys regulated molecules belong in large part to families associated with chemokines and their signaling partners. This novel bioinformatics tool has uncovered interactive pathways as well as functional relevance to further support MDA-Lys as a pro-inflammatory agent that can promote chemokine signaling and monocyte activation.

**MDA-Lys induces CCL11 protein in THP-1 cells.** CCL11, also known as eotaxin, plays a key role in coronary artery disease (22). However, its role in diabetic vascular disease has not been studied. Since our novel observations showed for the first time that MDA-Lys could induce mRNA and protein expression of CCL11 in monocytes, we evaluated it more extensively. The time course of CCL11 mRNA induction showed a peak at 8h (Fig.4B) in THP-1 cells and this was significant compared to equimolar MDA alone or NG (Fig.6A) suggesting that CCL11 induction was specific to MDA-Lys. Additional ELISAs to quantify CCL11 (Fig.6B) showed a significant increase (111.1 ± 41.5 pg/ml) in supernates from MDA-Lys treated cells relative to NG (7.3 ± 2.7 pg/ml).

**Increased CCL11 levels correlates with diabetic rat plasma MDA.** In order to determine in vivo relevance, we quantified MDA-Lys as well as CCL11 (eotaxin) levels in plasma from control and streptozotocin (STZ) injected diabetic rats (32 weeks) (10 control and 10 diabetic) using an ELISA method. MDA-Lys levels were significantly greater in diabetic rat plasma (225.7 ± 43.63 nmol/l) compared to control rat plasma (68.62 ± 14.33 nmol/l)(Fig-6C). Furthermore, in parallel, we observed a significant increase in eotaxin levels in diabetic rat plasma (231.15 ± 75.04 pg/ml) versus control non-diabetic (50.57 ± 30.12 pg/ml) (Fig-6D) suggesting that increased CCL11 levels in diabetes may be related to the increased MDA-Lys levels.

**MDA-Lys induces adhesion of monocytes to human vascular smooth muscle cells (HVSMC) and human umbilical vein endothelial cells (HUVECs).** Monocyte adhesion to ECs and VSMC are key pathogenic events associated with monocyte activation, sub-endothelial retention and atherosclerosis. To evaluate the functional role of MDA-Lys, we next examined whether pre-treatment of monocytes with MDA-Lys can enhance their adhesion to HVSMC or HUVECs. THP-1 cells were cultured in NG or with MDA-Lys in serum depleted medium for 24 h, and then allowed to adhere to monolayers of normal HVSMCs and HUVECs as described under Methods (Supplement). As seen in Fig. 7A and 7B, THP-1 cells treated with MDA-Lys adhered 2-3 fold more to HVSMCs and HUVECs relative to NG. Thus, elevated ALEs such as
MDA-Lys under diabetic conditions can enhance monocyte dysfunction. 

Identification of signaling pathways involved in MDA-Lys induced monocyte adhesion. RAGE ligands and HG can induced inflammatory genes and monocyte activation via enhanced oxidant stress, activation of NF-κB and AP-1, as well as key signaling kinases such as PKC, ERK1/2 and p38 MAPKs (14; 20; 23-25). Our current data show that MDA-Lys can also activate oxidant stress and NF-κB in monocytes which may involve kinase activation. We therefore examined the consequences of blocking some of these signaling pathways with specific inhibitors. Fig. 7C shows that MDA-Lys-induced monocyte binding to HVSMC was significantly blocked by the antioxidant NAC (100 µmol/l), as well as by p38MAPK inhibitor (SB, 5µmol/l), but not by inhibitors of ERK1/2 pathway (PD) or PKC (GFx). These results suggest that MDA-Lys-induced inflammatory genes, monocyte activation and adhesion involve the coordinated activation of multiple pathways, including oxidant stress and p38MAPK.

DISCUSSION

Recent studies suggest that, apart from AGEs, ALEs may also play significant roles in diabetic vasculopathy. However, although an immense amount of work has evaluated in vitro and in vivo functional roles of AGEs, very little is known about the mechanisms by which ALEs may exert their effects despite reports showing increased MDA-Lys in diabetic animal models (7, 10, 26). The role of MDA-Lys in diabetes-induced atherogenesis is also unclear. Monocytes are orchestrators of the inflammatory response and play key roles in the development of atherosclerosis.(27) We recently demonstrated that HG, or ligation of RAGE with AGEs or S100b in THP-1 monocytes or human blood monocytes lead to increased expression of several inflammatory genes and their receptors (14, 18-20, 23). Although the cellular effect of ALEs are believed to be similar to AGEs, this has not been tested to date. In the current study we have explored for the fist time the impact of MDA-Lysine on monocyte behavior by studying its effects on inflammatory gene expression and monocyte activation associated with diabetes. Synthetic MDA-Lys was used as a prototype ALE since it is elevated under diabetic conditions. We used 50µmol/l MDA-Lys since it gave the maximal induction of key inflammatory genes. There are very few reports of MDA-Lys levels in vivo. Miyata et al have reported about 20µmol/mol Lys of MDA in normal humans and about 40µmol/mol Lys in diabetics (34).

HG, AGEs and RAGE ligation increase intracellular oxidative stress in monocytes (13, 28) as manifested by the production of superoxide anion, a potent ROS. ROS function as important second messengers to modulate several downstream signaling molecules like MAPKs and transcription factors such as NF-κB and AP-1. These in turn can induce key inflammatory genes that promote monocyte activation, migration and sustained inflammation associated with several diabetic complications including atherosclerosis. We noted that MDA-Lys could significantly increase oxidant stress and superoxide production as well as NF-κB activation and inflammatory gene expression. In a candidate gene profiling approach, MDA-Lys increased the expression of key NF-κB dependent genes such as MCP-1, iNOS, RAGE, IP-10, CCR-2, IL-6, IL-8 and COX-2, that are associated with diabetic complications, inflammation and monocyte activation. Thus ROS signaling may mediate MDA-Lys induced NF-κB transactivation during diabetes, vascular dysfunction and atherosclerosis. Interestingly, IP-10 induction was not transcriptionally regulated suggesting that MDA-Lys might increase IP-10 mRNA
stability similar to that observed with the RAGE ligand, S100b (20).

In addition, we performed a focused profiling of cytokine, chemokine and related factors regulated by MDA-Lys in THP-1 cells by using cytokine antibody arrays, with subsequent follow-up validation of key candidates. Interestingly we noted significant increases in several factors that were primarily secreted including CCL11, CCL18, TNFSF14 and MCP-1 (Table1, Fig-3). A review of the literature showed that many of these are regulated by NF-κB (14, 19, 20, 29). Our data also showed increased NF-κB (but not AP-1 or Egr-1) activity supporting the notion that MDA-Lys-induced NF-κB activation can augment the expression of multiple cytokines implicated in monocyte activation.

On the other hand, while there was a 4-fold increase in CCL18 protein levels, there was no change in CCL18 mRNA levels suggesting that MDA-Lys may exert its effects not only by increasing the transcription of a subset of proinflammatory genes, but also by post transcriptional mechanisms and translational increase in protein synthesis of other genes. We also used bioinformatics pathway analyses (IPA) approach to determine whether ALEs can trigger key pathways and networks that modulate cellular function. This grouped the MDA-Lys induced genes/proteins into two types of high scoring pathways whose major functions were mainly related to inflammation, cell movement and cell-to-cell signaling (Table-1) with high significance associated with chemokine family proteins and their partners. These novel query tools clearly demonstrate that MDA-Lys can initiate a chemokine signaling program in monocytes which in turn can promote inflammation, cell migration, adhesion, extravasation, differentiation and vascular dysfunction.

Although both ALEs and AGEs are formed by Maillard reactions, it it not known whether both molecules share the same receptor, at least to some extent. MDA-Lys induced CCL11 mRNA accumulation was significantly attenuated in THP-1 cells pretreated with anti-RAGE antibody. However, under these conditions, TNFSF14 accumulation was not blocked, suggesting that MDA-Lys induced TNFSF14 mRNA is RAGE–independent and may involve an unidentified ALE receptor. Also, MDA-Lys could directly induce the expression of RAGE, but not the scavenger receptor CD-36, which is another AGE receptor (35). Reports show that ALEs such as MDA and CML are colocalized with macrophage scavenger receptor-A in atherosclerotic lesions (30). Thus MDA-Lys may act through multiple receptors and additional studies are needed to examine these.

Antibody array profiling revealed that MDA-Lys can upregulate interesting chemokines CCL11 (eotaxin), TNFSF14 and CCL18. Similar data was obtained in primary blood monocytes. Eotaxin is a novel chemokine with key emerging functions related to inflammation, atherosclerosis and associated diseases (22, 31). It can stimulate the directional migration, adhesion, accumulation, and recruitment of T lymphocytes during certain types of inflammation (22). Importantly, we noted in vivo relevance since plasma from diabetic rats had significantly elevated levels of not only MDA-Lys, but also eotaxin.

TNFSF14, a member of the TNF superfamily, is associated with and released as a soluble ligand upon platelet activation. TNFSF14 can induce inflammatory responses in monocytes and release chemokines. TNFSF14 has been implicated in atherogenesis, plaque destabilization and other inflammatory disorders involving leukocyte infiltration (32). CCL18 is a
chemokine that is induced during inflammation (33).

Evidence shows that HG, AGEs and S100b can increase monocyte adherence to VSMC and ECs (14, 18, 19), key early steps in the pathogenesis of atherosclerosis. Key factors that we noted to be induced by MDA-Lys, such as MCP-1, Eotaxin, IL-6, IL-8, β1 and β2-integrins and COX-2 are associated with monocyte activation, adhesion and migration. We observed that MDA-Lys treatment of monocytes led to 2-3 fold increase in their adherence to HVSMC and HUVEC. This increased adhesion was significantly blocked by inhibitors of p38 MAPK and oxidant stress. Kumagai et al (36) showed, that another lipid peroxidation derived electrophile, hydroxyl-nonenal (HNE), can induce COX-2 in macrophages and also activate p38MAPK. However, unlike MDA-Lys, HNE did not activate NF-κB or induce iNOS. Kanayama et al (37) showed that HNE upregulates CD36 expression, while our data shows that MDA-Lys had no effect on CD36 mRNA. Thus, MDA-Lys and HNE may act via different signaling mechanisms. Thus, ALEs such as MDA-Lys can induce key inflammatory genes in monocytes via oxidant stress, key signaling pathways (p38MAPK) and transcriptional factors (NF-κB) to enhance chemokine expression, monocyte adhesion, retention and foam cell formation.

Although our results are primarily derived with MDA-Lys, in unpublished results we noted that MDA-modified BSA elicits similar responses including eotaxin production. Taken together, these results provide, to our knowledge, the first evidence that MDA-Lys may enhance diabetic complications by inducing key pathologic genes and thus provide new insights into the actions of the excess MDA-Lys and ALEs seen in disease states. Future studies aimed at identifying ALE-specific receptors in target cells may uncover important therapeutic targets for diseases associated with dyslipidemia and enhanced ALEs such as diabetes and atherosclerosis.

FOOTNOTES

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REFERENCES


TABLE-1. Protein networks identified by pathway analysis

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<td>56</td>
<td>22</td>
<td>Immune Response, Cellular Movement, Hematological System Development and Function</td>
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<tr>
<td>2 ANG, AXL, BTC, BTN3A3 C10orf10, CCL8, CHST4, CIB2, CXCL13, DGCR6, FCGR1B, FCGR1C, GSTA4, H60, HRSP12, ICAM3, IFNG, IL6, IL1A, IL1F6, IL1F9, JUN, MAPK14, MYHS, NTF3, RAET1B, SLC28A1, SLC28A2, SPTA1, STAT3, TNF, TNFSF14, TNN, TRAFD1, UBR2</td>
<td>14</td>
<td>8</td>
<td>Cell-To-Cell, Signaling and Hematological System Development and Function</td>
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FIGURE LEGENDS

Figure 1. MDA-Lys induces oxidant stress and NF-κB activation in THP-1 cells. A. Fluorescence photomicrographs from control THP-1 cells (left panel), and THP-1 cells treated with 50 mol/l MDA-Lys (right panel) for 30 min exposed to either 1μmol/L H2DCFDA (DCF, for ROS, top panel) or 1μmol/L DHE (for superoxide, bottom panel) for 15 min. B. Bar graph showing significant 4 fold increase (P < 0.05 vs. NG) in ROS and 2 fold increase in superoxide formation (***P <0.01 vs NG) by MDA-Lys (mean ± SE, n = 3). C. EMSAs of nuclear extracts from NG or MDA-Lys treated cells incubated with radiolabeled oligonucleotides containing NFκB, AP-1 or Egr-1 consensus binding sites. Supershifts were performed with nuclear extracts of MDA-Lys treated cell extracts that were pretreated with p65 antibody. D. THP-1 cells were transiently transfected with 500 ng of p3xNF-kB reporter plasmid carrying luciferase gene under control of NF-kB consensus elements. After 12h, cells were treated with or without 50μmol/l MDA-Lys and cultured for an additional 7h. Results are from three individual experiments, each performed in triplicate. E. MDA-Lys induces phosphorylation of NF-κB p65. Lysates from THP-1 cells stimulated with MDA-Lys for 1 h were immunoblotted with antibodies to phospho-p65 or total p65 as internal control. F-H. Gene promoter transactivation by MDA-Lys. THP-1 cells were transfected with plasmids containing luciferase gene under the control of respective minimal human promoters of (F) IP-10 (–438/+97) (G) COX-2 (-860/+127) alone or with pCMV-mIκB, or (H) MCP-1 (-3011 to +37) alone or with pCMV-mIκB. After overnight recovery, cells were treated with MDA-Lys for 8h, and luciferase activities determined. Values shown are normalized to 50μg protein. Mean ± SE of three independent experiments (*P<0.01 vs NG, **P<0.05 vs NG).

Figure 2. Analysis of MDA-Lys-induced candidate genes. Relative RT-PCRs were performed with total RNA isolated from THP-1 cells treated with or without MDA-Lys for 1 to 24 h, using gene-specific primers (Table-S1). 18S RNA primers were included in each PCR reaction as internal control. A. Ethidium bromide-stained agarose gels of RT-PCR products. NG, indicates control cell grown for 24h. B. Bar graph showing significant induction of β1 and β2-integrins, CCR2, iNOS, COX-2, RAGE, IP-10, IL-6, IL-8 and MCP-1 mRNAs at 4, 8 or 24 hr. Values shown are mean ±SE of three independent experiments. *P < 0.001, **P<0.05 vs NG.

Figure 3. Cytokine antibody array analysis of MDA-Lys treated THP-1 cells. Conditioned medium from THP-1 monocytes cultured under either NG (5.5 mM) or MDA-Lys (50μmol/l) conditions for 24 h were hybridized to human cytokine antibody arrays. Relative percentage of spot intensities in the membranes were measured and normalized to NG. Values are average of data from three independent experiments. Cytokines showing significant increases (*P, ≤0.05) are shown in the bar graph.

Figure 4. A. Role of RAGE receptor in MDA-Lys induced CCL11, CCL18, and TNFSF14 in mRNAs. THP-1 cells were pretreated with or without 70μg/ml anti-RAGE antibody for 1 h followed by 50μmol/l MDA-Lys for 4 h. mRNA levels analyzed by RT-PCR with 18S internal control. B. Bar graph showing time course induction of CCL11, CCL18 and TNFSF14 mRNAs by real time qRT-PCR using gene-specific primers and
normalized with GAPDH as internal control. C. Effect of MDA-Lys on CCL11, CCL18 and TNFSF14 mRNA expression in peripheral blood primary human monocytes as analyzed by RT-PCR with 18S internal control. D. Agarose gel of RT-PCR products of TNFSF-14, CCL11 and CCL18 mRNA amplification from THP-1 cells treated with or without methylglyoxal modified bovine serum albumin (AGE) for 8h.

Figure 5. Ingenuity Pathway Analysis identifies key interactive networks among proteins and genes regulated by MDA-Lys in THP-1 cells. A. The network is displayed schematically as nodes (genes/gene products) and edges (solid or dotted lines, depicting biological relationships between the nodes). Filled nodes are identified targets and open ones are predicted pathway partners. Edges for protein regulations such as phosphorylation and other modifications are removed for simplicity. This network was generated by the software based on highest scores obtained from a total of 22 differentially expressed focus proteins noted in our experiments (Supplement Table 2). In the current study, a score of 14 or higher was used to select significant biological networks regulated by MDA-Lys. A highly significant score of 56 was obtained (Table-1). B. The network score is next displayed as the negative log of the P value, indicating the likelihood of the focus molecules in a network being found together due to random chance. Therefore, –log values of 2 have at least 99% confidence of not being generated by chance alone as seen for chemokine signaling.

Figure 6. MDA-Lys induces specific and significant induction of CCL11. Total RNA from multiple sets of MDA-Lys, MDA and NG grown THP-1 cells were used to perform real time qPCRs using CCL11 specific primers and GAPDH primers. A. Eotaxin levels were calculated by normalizing to internal control GAPDH and results expressed as fold over NG. Values shown are mean SE of three independent experiments. *P < 0.012. B: Conditioned medium supernatants of THP-1 cells treated with NG or MDA-Lys were assayed for secreted CCL11 (eotaxin) levels by specific ELISA. Results shown are mean ± SE from three independent experiments run in triplicate *P< 0.004. C: Quantification of protein cross-linked MDA in plasma from control and STZ treated rats (N=10) *P< 0.003. D: Rat plasma samples assayed for secreted CCL11 levels by specific ELISA. Results shown are mean ± SE from four independent rats run in triplicate *P< 0.003.

Figure 7. A. MDA-Lys treatment increases monocytes adhesion to HVSMC and HUVECs. THP-1 cells were cultured with or without MDA-Lys for 12 h and then labeled with fluorescent dye Calcein-AM for 15min at 37°C. Labeled THP-1 cells were allowed to adhere to either HUVEC or HVSMC monolayers in 24 well culture dishes. After careful washing, specifically bound monocytes were counted as described (19). Results are expressed as number of monocytes bound per high power field. Bar graph B shows mean ± SE from 3-5 experiments (*P<0.005, ** P < 0.01 versus respective controls). C. THP-1 cells were treated for 12 h with the indicated inhibitors or corresponding vehicle and then treated with or without MDA-Lys and then binding to HVSMC examined as above. Results shown are mean ± SEM (n=4; *P<0.01 versus respective control). PD; 2’-Amino-3’methoxyflavone (PD-98059), SB; 4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol, (SB202190), bis-indolylmaleimide; (GFX), and NAC; N-acetyl-cysteine
FIGURE-1

(A)  (B)  (C)

(D)  (E)  (F)

(G)  (H)

MDA-Lys Induced Cytokine Expression
FIGURE-2
MDA-Lys Induced Cytokine Expression

FIGURE-4

(A) MDA-Lys Induced Cytokine Expression

(B) Anti RAGE Antibody

(C) NG MDA-Lys

(D) Anti RAGE Antibody

Fold Induction

Time (h) 24 2 4 8 24 8 8

CCL11

Fold Induction

Time (h) 24 2 4 8 24 8 8

CCL18

Fold Induction

Time (h) 24 2 4 8 8

CCL18
FIGURE-5

(A) [Diagram showing interactions between different molecules labeled with symbols for enzyme, cytokine, direct interaction, transcription regulator, peptidase, inhibition, G-Protein coupled receptor, transmembrane receptor, indirect interaction, other, and growth factor.]

(B) [Bar chart showing log (significance) for Chemokine Signalling, Integrin Signalling, IL-2 Signalling, and IL-6 Signalling with bars at log (significance) of 0.5, 1.0, 1.5, and 2.0 with a threshold indicated.]
FIGURE-6

(A) Fold induction

(B) Eotaxin (pg/ml)

(C) MDA-Lys (nmol/l)

(D) Eotaxin (pg/ml)
FIGURE-7

(A) HUVEC

(B) HVS MC

(C) Number of cells/field

MDA-Lys Induced Cytokine Expression