Advanced Glycation End Products Stimulate an Enhanced Neutrophil Respiratory Burst Mediated Through the Activation of Cytosolic Phospholipase A₂ and Generation of Arachidonic Acid

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Background—Advanced glycation end products (AGEs) enhance NADPH oxidase, and hence respiratory burst activity, of stimulated neutrophils. They are thus potentially vasculopathic, especially in diabetes, uremia, and aging, in which AGEs classically accumulate. We investigated the underlying mechanisms.

Methods and Results—Neutrophils prelabeled with [3H]arachidonic acid display increased [3H]arachidonate release on exposure to AGE-albumin over exposure to albumin alone (by 151±6%/H1100616%, P<0.01). Arachidonic acid (AA) itself seems to mediate the AGE-enhanced neutrophil respiratory burst (ascertained by chemiluminescence). Inhibitors of the cyclooxygenase pathway (indomethacin) and lipoxygenase pathway (MK-886) do not impair this AGE effect, excluding a contribution from AA metabolites. Cytosolic phospholipase A₂ (cPLA₂) controls AA generation. Its inhibition by methyl arachidonyl fluorophosphonate abrogates the AGE-enhanced activated neutrophil respiratory burst, and it is demonstrably stimulated in AGE-exposed neutrophils, as evidenced by isoform gel-shift and an increasingly membrane-translocated state in Western blots of neutrophil subfractions. Inhibition of other PLA₂ isoforms, secretory PLA₂ and calcium-independent PLA₂, by manoalide and haloenol-lactone suicide substrate, respectively, does not affect this effect of AGEs relative to inhibitor-treated controls. The thiol antioxidant NAC reduces activation of cPLA₂ (assessed by isoform gel-shift and membrane translocation), production of AA in AGE-albumin–exposed neutrophils ([H]release reduced to 104±17%, P=0.94 compared with albumin-exposed neutrophils), and the AGE-augmented neutrophil respiratory burst.

Conclusions—AGE augmentation of the activated neutrophil respiratory burst requires AA generation, through which neutrophil NADPH oxidase may be upregulated, enhancing reactive oxygen species output. AA is generated by cPLA₂, which may be stimulated through an AGE-activated redox-sensitive pathway. (Circulation. 2003;108:1858-1864.)

Key Words: glycosylation end products, advanced phospholipases arachidonic acid free radicals NADPH oxidase

The accumulation of advanced glycation end products (AGEs) resulting from the nonenzymatic reaction of amino acids with aldose sugars, is implicated in pathological conditions, particularly the vascular disease encountered in diabetes mellitus, uremia, and aging.¹⁻³ This may be mediated by oxidative stress⁴⁻⁵; the aforementioned states are associated with increased oxidative stress.⁶⁻⁸ The role of oxidative stress in the pathophysiology of vascular disease is already established.⁹ One mechanism by which AGES may induce oxidant stress is by enhancing free radical generation from neutrophils. AGES act as neutrophil coagonists, having no direct, discernible effect on neutrophil function, yet augmenting the respiratory burst induced by a secondary stimulus.¹⁰ This is dependent on neutrophil NADPH oxidase, a multi-component enzyme that generates superoxide.¹¹

The mechanism governing this AGE effect has not been characterized, although its initiation is probably receptor-mediated. Receptors capable of binding AGES have been described, of which the receptor for AGES is the best characterized,¹² with a common feature of its ligation being the activation of redox-sensitive pathways.¹³ Also of particular pertinence regarding the coagonist action of AGES on the NADPH oxidase are similar reports ascribing a facilitatory action on NADPH oxidase to arachidonic acid (AA), an unsaturated fatty acid produced by phospholipase A₂ (PLA₂) enzymes.¹⁴,¹⁵

In seeking to understand how AGES may contribute to vascular pathology, we investigated the costimulatory actions of AGES on the neutrophil NADPH oxidase with respect to generation of reactive oxygen species (ROS). We found that...
AGE-induced generation of AA, produced by cytosolic PLA₂ (cPLA₂), is the basis of this phenomenon and may be regulated by a redox-sensitive mechanism.

**Methods**

**Materials**

Bis-N-methylacridinium nitrate (lucigenin), fMLP, glucose, indo-methacin, N-acetylcysteine (NAC), Percoll and tissue culture medium 199 (TC199) were all from Sigma-Aldrich. Haloenol-lactone suicide substrate (HELSS), manoalide, and methyl arachidonyl fluorophosphonate (MAFP) were from Biomol. MK-886 was from Calbiochem, 20% endotoxin-free human serum albumin (HSA) was from BioProducts Laboratory, and [3 H]arachidonic acid ([3 H]-AA) was obtained from Amersham.

**Preparation of AGE-Albumin**

AGE-albumin was prepared by preincubation of endotoxin-free HSA (20%) with 1 mol/L glucose at 37°C for 12 weeks in 100 mmol/L phosphate (pH 7.4). Control albumin was produced by incubation of the same HSA preparation under identical conditions but without the glucose. After incubation, HSA preparations were dialyzed against PBS and 0.9% sodium chloride for 24 hours before sterilization by filtration.

**Neutrophil Isolation and Fractionation**

Fresh citrated blood was obtained from healthy adult volunteers after they had given informed consent. Neutrophils were separated by dextran sedimentation and purification on a Percoll gradient.10 Membrane and cytosol fractions were prepared according to the method of Levy and Malech.16 Briefly, neutrophils were centrifuged down, resuspended in relaxation buffer (in mmol/L: 10 KCl, 3 NaCl, 3.5 MgCl₂, 1.25 EGTA, 10 HEPES, pH 7.4, containing 1 mmol/L PMSF, 10 μmol/L leupeptin, 10 μg/mL aprotinin, 1 mmol/L Na₃VO₄, and 25 mmol/L NaF), snap-frozen in liquid nitrogen, defrosted, and sonicated 3 times for 15 seconds on ice. The postnuclear supernatant was centrifuged at 150 000g for 60 minutes (Beckman Optima Max Ultracentrifuge) to obtain a cell membrane pellet and cytosol supernatant. Membrane pellets were resuspended in the above relaxation buffer.

**Neutrophil Stimulation and Inhibition**

Mechanical stimulation of neutrophils was by multiple passages through a standard 1-mL pipette tip.10 Chemical stimulation was in the form of fMLP 100 nmol/L.17 When we investigated the effects of various enzyme inhibitors on the AGE-augmented neutrophil ROS burst, neutrophils were preincubated with the respective inhibitors, after which neutrophils were spun down and resuspended in inhibitor-free buffer (containing AGE-albumin/HSA) before stimulation.

**Detection of Reactive Oxygen Species**

ROS detection was based on lucigenin enhanced chemiluminescence (ECL)18 recorded on an EG&G Berthold microplate luminometer LB96V. All experiments were performed at 37°C. Lucigenin 50 μmol/L was made up in a balanced salt solution containing (in mmol/L) 140 NaCl, 15 HEPES, 5 KCl, 5 glucose, 1.8 CaCl₂·2H₂O, and 0.8 MgSO₄·7H₂O (pH 7.4). All experiments were performed in triplicate with 10⁵ cells per well.

**Western Blotting**

Cytosol and membrane fractions (100 μg protein) were resolved on 10% (wt/vol) SDS-polyacrylamide gels. The resolved proteins were...
Detection of AA Production
Labeling of neutrophils with [3H]-AA and the release of radiolabeled AA were performed according to the method of DiPersio et al. Briefly, the cells were incubated with 2.5 μCi/mL [3H]-AA for 45 minutes at 37°C. Those cells requiring further treatment with MAFP, NAC, or HELSS were incubated for a further 60 minutes in TC199 with 1% BSA. Cells were then washed 3 times in TC199 and incubated for 30 minutes with AGE-albumin or albumin at 200 μg/mL in TC199. After this final incubation, cells were centrifuged, and an aliquot of the supernatant was counted by liquid scintigraphy.

AA Binding by AGE-Albumin
To examine whether binding of AA was different between AGE-albumin and control HSA, we incubated and equilibrated 1 μCi/mL [3H]-AA for 6 hours at 37°C with both AGE-albumin and control HSA and then measured the free fraction of [3H]-AA by ultrafiltering the solutions on 30-kD cutoff Microcon filter devices (Millipore). The ultrafiltrates were counted by liquid scintigraphy.

Data Analysis
Chemiluminescence values are presented as relative light units per minute (RLU/min) (means with SEMs). For analysis of total ROS production (RLU), we calculated the area under the curve (cutoff at 6 minutes). Comparisons were with 2-tailed t tests or 1-way ANOVA with Tukey’s post hoc analysis as appropriate (SPSS Inc).

Results
AGE augmentation of the stimulated neutrophil respiratory burst is abolished by inhibitors of cPLA2 and by NAC. We previously demonstrated the ability of AGEs to augment the activated neutrophil respiratory burst. Here, we investigated the effects of manipulating both AA production and intracellular redox status on this particular property of AGEs. AA is hydrolyzed from membrane glycerophospholipids by PLA2, of which different isoforms exist. As shown previously, the respiratory burst was augmented in the presence of AGE-albumin compared with control albumin, after mechanical or chemical (fMLP) stimulation (Figure 1, A–E). This augmented response was abolished by a 30-minute preincubation of neutrophils with the minimum effective dose of either of 2 cPLA2 inhibitors, MAFP (50 nmol/L) and mepacrine (10 μmol/L), both being maximal effective doses. However, a 60-minute preincubation with other direct free-radical scavengers (ascorbic acid, acetylsalicylic acid, and tiron) produced negligible effects (data not shown), and inhibition of other PLA2 isoforms (secretory PLA2 by mannoalide, calcium-independent PLA2 by HELSS, both being maximal effective doses)
did not result in significant reduction of the AGE-augmented neutrophil respiratory burst compared with inhibitor-treated controls (Figure 1, C and D). Higher concentrations of HELSS damaged cells, reducing their viability (data not shown).

AGE-Exposed Neutrophils Exhibit Enhanced Liberation of AA, Which Is Abrogated by Inhibitors of cPLA2 and NAC

To confirm that AGE coagonist activity on neutrophil NADPH oxidase is mediated through AA, we assessed AA production. Neutrophils prelabeled with $^{3}$H-AA were incubated for 30 minutes in AGE-albumin or albumin (200 µg/mL). AGE-albumin--exposed neutrophils exhibited a higher rate of AA release than albumin-exposed neutrophils (151±16%, P<0.01) [Figure 2A]. This enhancement was abrogated by preincubation of neutrophils for 30 minutes with a minimum effective dose (50 nmol/L) of MAFP, an inhibitor of cPLA2, before AGE-albumin/albumin exposure (Figure 2A), confirming the role of cPLA2 in AA synthesis in this context. Because the interaction of AGEs with some cellular receptors has been noted to activate redox-sensitive pathways,13 we also sought to establish whether redox-sensitive pathways might govern the AGE-induced increase in neutrophil AA production. We manipulated intracellular levels of reduced glutathione using a 60-minute preincubation of neutrophils with NAC, a precursor of glutathione. Subsequent exposure of neutrophils to AGE-albumin did not result in any significant increase in AA production over control cells (104±17%, P=0.94) (Figure 2A). In another series of experiments, we confirmed that the enhancing effect of AGE-albumin on neutrophil AA release was not affected by pretreatment with the calcium-independent PLA2 antagonist HELSS (100 nmol/L) (Figure 2B).

**Table 1. Quantitative Comparison of cPLA2 (Nonphosphorylated) in Different Neutrophil Fractions After Exposure to AGE-Albumin/Albumin**

<table>
<thead>
<tr>
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<th>Cytosol</th>
<th>Membrane</th>
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<tbody>
<tr>
<td></td>
<td>AGE-Albumin</td>
<td>Albumin</td>
</tr>
<tr>
<td>cPLA2</td>
<td>0.53 (0.02)</td>
<td>0.75 (0.05)</td>
</tr>
<tr>
<td>cPLA2 (+NAC)</td>
<td>0.56 (0.06)</td>
<td>0.72 (0.02)</td>
</tr>
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</table>

The phosphorylated cPLA2 band is seen only in the membrane fraction of neutrophils exposed to AGE-albumin. Mean (SEM) and P values (by Tukey’s test) are quoted for 5 experiments.

*For both the cytosolic and membrane fractions, P<0.01 between groups by ANOVA.

**Figure 4. Acute neutrophil ROS production detected by lucigenin ECL in presence of AGE-albumin/albumin (200 µg/mL) and/or varying doses of AA and in response to mechanical or chemical (fMLP) stimuli. A, Total neutrophil ROS production (over a period of 6 minutes) after stimulation in presence of AGE-albumin (200 µg/mL), albumin (200 µg/mL), or albumin (200 µg/mL) plus varying doses of AA (P<0.01 between groups by ANOVA). B, Peak neutrophil ROS production after stimulation in presence of AGE-albumin (200 µg/mL), albumin (200 µg/mL), or albumin (200 µg/mL) plus various doses of AA (P<0.01 between groups by ANOVA). Each recording is mean of 3 separate aliquots of 10^5 cells measured simultaneously, and results are representative of multiple experiments. Total ROS production was measured in RLU and peak ROS production in RLU/min.
The differences in AA release may have been a result of altered binding or trapping of AA by AGE-albumin compared with control HSA. We therefore measured the AA counts in ultrafiltrates of buffer containing either AGE-albumin or control HSA with 10 μCi/mL [3H]-AA. In 5 experiments, 200 μg/mL AGE-albumin bound 79.3±0.12% of the available [3H]-AA, compared with 200 μg/mL HSA, which bound 81.0±0.13% of the AA (P<0.001). This small difference, although significant, would have had minimal effect on the trapping of AA released from the cells and would not have accounted for the increased AA release in AGE-albumin–treated cells, because AGE-albumin bound less of the labeled AA.

AGEs Mediate Activation of cPLA2
Activation of the enzyme cPLA2 requires its phosphorylation and translocation from the cytosol to the plasma membrane.25 After transient exposure to AGE-albumin/albumin followed by resuspension in relaxation buffer, neutrophils were rapidly fractionated into membrane and cytosolic components. Immunoblots revealed increased cPLA2 in the plasma membrane fractions, with a corresponding decrease in the cytosolic fractions, in AGE-albumin–exposed compared with albumin–exposed neutrophils (Figure 3 and the Table). In addition, the presence of a phosphorylated cPLA2 moiety in the membrane fraction derived from cells exposed to AGE-albumin was revealed through reduced electrophoretic mobility or gel-shift (Figure 3). Such AGE-induced cPLA2 phosphorylation and increased membrane translocation was abolished by a 60-minute preincubation of neutrophils with 10 mmol/L NAC (Figure 3 and the Table).

AA Mimics AGE-Induced Augmentation of the Stimulated Neutrophil Respiratory Burst
Because cPLA2 inhibitors nullified AGE-induced AA release and AGE-enhanced ROS release from stimulated neutrophils, we investigated ROS production after direct addition of AA to neutrophils. Neutrophil ROS release (both total and peak) in response to stimuli was enhanced in the presence of AA ≥50 nmol/L (average enhancement of total ROS production by 157% with mechanical stimulus and 163% with fMLP stimulus), and the magnitude of this enhancement was similar to that induced by AGE-albumin itself (average enhancement of total ROS production by 168% with mechanical stimulus and 152% with fMLP stimulus) (Figure 4, A and B).

AA and Not One of Its Metabolites Is Responsible for Augmenting the Stimulated Neutrophil Respiratory Burst
To determine whether the facilitatory role of AA in NADPH oxidase activation might be mediated through its downstream metabolites, we assessed the effects of specific inhibitors of the cyclooxygenase and lipoxygenase pathways on AGE-augmented neutrophil ROS production. The cyclooxygenase inhibitor indomethacin23 and the lipoxygenase inhibitor MK-886 26 did not attenuate the differential enhancement of ROS production caused by AGE-albumin in response to mechanical/chemical stimuli (Figure 4, A and B).

NAC Does Not Affect the AA-Enhanced Response of Neutrophils to Mechanical or Chemical Stimuli
The direct effect of AA on ROS production of neutrophils with both mechanical and chemical stimuli was tested in the
absence and presence of NAC pretreatment (10 mmol/L). NAC pretreatment had no effect on the AA enhancement of ROS production with either stimulus (P < 0.001 by Tukey’s test for both stimuli between control and AA-treated cells in the absence or presence of NAC, n = 8 experiments, Figure 6).

Discussion
Evidence from this study suggests how AGEs might enhance neutrophil NADPH oxidase activity and contribute to the increased vascular oxidant stress and cardiovascular disease in diabetic, uremic, and elderly patients. AA seems central to this process, mimicking the coagonist activity of AGEs. This may reflect its ability to alter the structural conformation and functional efficiency of the p47 subunit of NADPH oxidase or action on other intracellular components vital to the function of the oxidase. The proportionally small increment in AA release induced by AGE exposure is consistent with a catalytic function.

AGE-induced AA production seems to be dependent on cPLA₂ activation, which becomes phosphorylated and membrane-translocated on exposure of neutrophils to AGEs. Calcium facilitates this process; indeed, membrane translocation may take place in the presence of calcium alone without phosphorylation, explaining the significant presence of nonphosphorylated cPLA₂ in the other membrane fractions. In HELSS-treated neutrophils, AGE-albumin–stimulated AA release was unaffected, making it unlikely that calcium-independent PLA₂ is involved in these effects. In addition, others have documented that calcium-independent PLA₂ is not involved in the fMLP stimulation of neutrophil AA release. In contrast, cPLA₂ has a major role in AA release from fMLP-stimulated neutrophils, and this is associated with translocation of cPLA₂ to membranes and a retarded electrophoretic mobility consistent with enhanced phosphorylation.

There is a hint that redox-sensitive mechanisms may also play a role in transducing the effect of AGE on neutrophil NADPH oxidase, but few clinical trials have demonstrated a reduction in vascular events from antioxidant supplementation. This may be partly a result of inadequate antioxidant plasma levels, but our unpublished data on free radical scavengers showed no attenuation of AGE-augmented neutrophil ROS production. The exception was NAC, which reduced the final ROS output but more specifically abrogated the AGE-induced differential increase in ROS output. This superiority of NAC may relate to its role as a glutathione precursor, the principal intracellular free radical scavenger, because glutathione depletion occurs in AGE-exposed cells. Reduced glutathione may inhibit intracellular redox-sensitive pathways in which trace quantities of free radicals mediate signaling. From the ability of NAC to reduce AA production and cPLA₂ translocation, this putative redox signaling is likely to occur upstream of cPLA₂ activation. Activation of redox signaling by AGEs has been already described in the

Figure 7. A putative pathway by which AGEs may enhance stimulatory burst activity of neutrophil NADPH oxidase. Possible ligation of a receptor by AGEs generates intracellular superoxide (O₂⁻), which through a redox-sensitive pathway involving 1 or more protein kinases results in phosphorylation, membrane translocation, and subsequent activation of cPLA₂. cPLA₂ hydrolyzes AA from membrane glycerophospholipid, which then conformationally alters p47 subunit of NADPH oxidase (subunits illustrated) to increase functional efficiency of oxidase complex.
context of ligation for receptor for AGE by AGEs, and it remains likely that a receptor-mediated event is the initiating focus in the cascade of events described above (Figure 7).

Further work is needed to determine the role for receptor ligation in the initiation of events and to delineate mechanisms by which redox signaling results in cPLA2 activation, although many candidate kinases exist. Unraveling such intracellular pathways may widen the number of potential therapeutic options when targeting vascular oxidant stress and disease.

Acknowledgments
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