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Receptor for Advanced Glycation Endproducts mediates proatherogenic responses to periodontal infection in vascular endothelial cells

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Abstract

Objective—A link between periodontal infections and an increased risk for vascular disease has been demonstrated. *Porphyromonas gingivalis*, a major periodontal pathogen, localizes in human atherosclerotic plaques, accelerates atherosclerosis in animal models and modulates vascular cell function. The receptor for advanced glycation endproducts (RAGE) regulates vascular inflammation and atherogenesis. We hypothesized that RAGE is involved in *P. gingivalis*'s contribution to proatherogenic responses in vascular endothelial cells.

Methods and Results—Murine aortic endothelial cells (MAEC) were isolated from wild type C57BL/6 or RAGE^{−/−} mice and were infected with *P. gingivalis* strain 381. *P. gingivalis* 381 infection significantly enhanced expression of RAGE in wild-type MAEC. Levels of proatherogenic advanced glycation endproducts (AGEs) and monocyte chemoattractant protein 1 (MCP-1) were significantly increased in wild-type MAEC following *P. gingivalis* 381 infection, but were unaffected in MAEC from RAGE^{−/−} mice or in MAEC infected with DPG3, a fimbriae-deficient mutant of *P. gingivalis* 381. Consistent with a role for oxidative stress and an AGE-dependent activation of RAGE in this setting, both antioxidant treatment and AGE blockade significantly suppressed RAGE gene expression and RAGE and MCP-1 protein levels in *P. gingivalis* 381 infected human aortic endothelial cells (HAEC).

Conclusion—The present findings implicate for the first time the AGE-RAGE axis in the amplification of proatherogenic responses triggered by *P. gingivalis* in vascular endothelial cells.

Keywords

RAGE; infection; *P. gingivalis*; periodontitis; endothelial cells; vascular inflammation; atherosclerosis

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1. Introduction

The relationship between infection, inflammation and atherosclerosis has been highlighted in recent years [1]. Periodontal diseases are chronic infections affecting the supporting structures of teeth with a high prevalence in most populations [2]. A link between periodontal infections and atherosclerosis-related vascular disease has been demonstrated [3]. The major periodontal pathogen, *Porphyromonas gingivalis*, has been shown to gain access to the systemic circulation, accelerate atherosclerosis in animal model studies, invade vascular cells and induce proatherogenic and procoagulant responses [4–11].

In addition to having important roles in body homeostasis, endothelial cells (ECs) serve as regulators of lipid transport and transduce inflammatory signals. The ability of *P. gingivalis* to interact with ECs is of importance, as EC injury leading to dysfunction is crucially involved in vascular inflammation and atherogenesis [1].

The multiligand receptor for advanced glycation endproducts (RAGE) is expressed in multiple cell types relevant to atherogenesis, including ECs, and its genetic deletion in atherosclerosis-prone mice has been shown to suppress vascular injury and atherosclerotic plaque formation [12]. Hyperglycemia, inflammation and oxidative stress drive the generation of RAGE and its ligands, such as advanced glycation endproducts (AGEs), high-mobility group box 1 (HMGB1) and S100/calgranulins, and RAGE confers its impact and that of its ligands via signal transduction pathways [13]. Previous studies have revealed a role for RAGE and its ligands in the pathogenesis of diabetes-associated periodontitis via amplification of the inflammatory response to the bacterial challenge and delayed bone healing [14,15].

Our present studies aim to explore mechanisms underlying accelerated atherogenesis upon *P. gingivalis* infection and are based on the hypothesis that RAGE is involved in the proatherogenic responses and endothelial activation elicited by this periodontal pathogen.

2. Methods

2.1 Animals, cells and bacterial strains

All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University. Mice were housed in a pathogen-free environment and allowed free access to normal rodent chow and water. Male C57BL/6 were purchased from Jackson Laboratories (Main Harbor, ME). Homozygous RAGE^{-/-} mice were backcrossed for more than 12 generations into C57BL/6 background. Genomic DNA was isolated from tail biopsies and PCR was used to identify the deficiency of the RAGE gene. Mouse aortic endothelial cells (MAEC) were isolated from 6 to 8 week old C57BL/6 or RAGE^{-/-} mice, and maintained in DMEM/F12HAM medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT) as previously described [16]. Cells were serum-starved overnight prior to infection and were maintained in serum-free medium after infection. Primary human aortic endothelial cells (HAEC, Lonza, San Diego, CA) were maintained in endothelial cell medium 2 (EGM-2, Lonza) with a serum concentration of 2% and other supplements provided by the supplier. *P. gingivalis* FDC381 and DPG3, its fimbriae-deficient mutant, were grown in anaerobic chambers at 37°C on blood agar plates without and with erythromycin, respectively (Anaerobe Systems, Morgan Hill, CA). Bacterial suspensions were prepared in phosphate buffered saline without Mg²⁺/Ca²⁺ (PBS) using established growth curves and spectrophotometric analysis.

2.2 Infection of aortic endothelial cells with *P. gingivalis*

MAEC or HAEC were plated (1.5×10^5 /well) in 6-well plates (Corning, Acton, MA) for 24 hours, and infected in antibiotic-free medium with *P. gingivalis* 381 or DPG3 for 90 minutes

(multiplicity of infection [MOI] of 1:100, calculated based on the number of cells per well when seeded), or left uninfected. Cells were then washed with PBS and maintained for 6 or 24 hours. Supernatants and cell lysates (prepared using M-PER reagent, Thermo Scientific, Waltham, MA) were stored at -70°C until further analysis.

2.3 AGE and MCP-1 ELISA

To measure AGE levels in cell supernatants, an enzyme linked immunosorbent assay (ELISA) was performed as previously described [17]. Briefly, samples were first incubated with blocking buffer (5% GSA, 1% BSA in 0.1 M PBS, pH 7.4) for 1 hour at room temperature and then an affinity-purified chicken anti-AGE antibody was used for 3 hours (Thermo Scientific, 1:100 in blocking buffer). The secondary antibody (anti-chicken IgG, Sigma) was diluted 1:10000 and used for 1 hour at room temperature. Signals were developed and read at 490 nm. Ribose glycosylated albumin was used to prepare the standard curve. Results are given in AGE units, established elsewhere (calculated as ng AGE-BSA/mg soluble protein) [18]. Levels of mouse and human MCP-1 were determined in cell culture supernatants by commercially available ELISA kits (Bender MedSystems, Burlingame, CA) according to the manufacturer's instructions. In blocking experiments, HAEC were preincubated with anti-RAGE IgG (70 $\mu\text{g}/\text{ml}$), or anti-AGE IgG (15 $\mu\text{g}/\text{ml}$), or aminoguanidine (Sigma, 200 $\mu\text{mol}/\text{L}$) for 2 hours [19], or with *N*-acetyl-L-cysteine (NAC, Sigma, 10 mmol/L), or diphenylene iodonium (DPI, Sigma, 30 $\mu\text{mol}/\text{L}$) for 1 hour and then infected with *P. gingivalis* 381 for 90 minutes. Aminoguanidine is an established AGE formation inhibitor, NAC is a well characterized thiol-containing antioxidant and DPI is an inhibitor of flavine-binding proteins such as NADPH oxidase, the primary source of reactive oxygen species (ROS) in ECs. Non-specific IgG antibody (Alpha Diagnostic, San Antonio, TX, 15 $\mu\text{g}/\text{ml}$) was used as a negative control. Following the PBS wash, blocking agents were reintroduced until the 6 hour time point when supernatants were collected for MCP-1 level assessments.

2.4 Immunoblotting and Real Time PCR

Lysates from cultured cells were used for detection of RAGE (GeneTex, Irvine, CA), high mobility box 1 (HMGB1, Abcam, Cambridge, MA) and β -actin (BD Bioscience) IgG. HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology) were used to identify sites of binding of the primary antibody. Densitometric analysis was performed using the ImageJ program (version 1.3.8x, NIH, Bethesda, MD). RNA was isolated from HAEC using Trizol (Invitrogen, Carlsbad, CA), followed by reverse transcription using the AffinityScript Reverse Transcriptase (Stratagene, Santa Clara, CA). Real Time PCR was subsequently performed for human RAGE and MCP-1 using Gene Expression Assays (Applied Biosystems, Foster City, CA), normalized to the human GAPDH probe set (Applied Biosystems) and analyzed on an Mx3005P Real Time PCR device (Stratagene). Data were analyzed using the comparative Ct method.

2.5 Data and statistical analysis

All experiments were performed in duplicate wells for each condition and repeated at least three times. Data are presented as mean $\pm$ S.D. or as mean fold difference from the non-infected control $\pm$ S.D. Representative experiments of 3 independent runs are shown for the immunoblotting studies. Statistical analysis of the murine cell data was performed by means of two-way ANOVA with the type of infection (no infection, *P. gingivalis* 381 or DPG3) and the status of RAGE expression in the endothelial cells (from C57BL/6 or RAGE $^{-/-}$ mice) or the time point tested (6 or 24 hours after infection) as the two independent variables. Analysis of data from experiments with the human cells was performed by one-way ANOVA with the type of infection or treatment as the independent variable. Pair-wise comparisons between the

different groups were subsequently carried out using a t-test and the post-hoc Ryan-Einot-Gabriel-Welsch multiple range test. P values <0.05 were considered statistically significant.

3. Results

3.1 RAGE levels in primary MAEC upon infection with *P. gingivalis*

It is well established that RAGE is expressed in endothelial cells and that its activation leads to cellular injury and inflammation [12]. We first explored whether *P. gingivalis* infection further affects levels of RAGE in C57BL/6 MAEC. The effect of infection depended on the time point tested (two-way ANOVA $p=0.0140$). As seen in Figure 1a, *P. gingivalis* 381 infection increased RAGE expression in C57BL/6 MAEC by 2.4-fold 6 hours after infection compared to the non-infected group ($p=0.0122$, $n=3$). At 24 hours (Figure 1b) the magnitude of the increase was smaller, 1.3-fold compared to control ($p=0.0128$, $n=3$). As the major fimbriae of *P. gingivalis* are required for the pathogen's adhesion to/invasion of host cells and for virulence, we also tested the effects of its fimbriae-deficient mutant, DPG3. We found a significant increase in RAGE expression in DPG3-infected MAEC compared to non-infected cells at 6 hours (2.4-fold, $p=0.0108$, $n=3$), but no effect at 24 hours ($p=0.7634$, $n=3$).

3.2 MCP-1 production by MAEC upon bacterial infection

The effect of infection type on the production of the proinflammatory chemokine MCP-1 depended on the status of RAGE expression in the endothelial cells (two-way ANOVA $p=0.0029$). In MAEC from C57BL/6 mice the effect of infection type also depended on the time point tested (two-way ANOVA $p<0.0001$).

Pair-wise comparisons specifically revealed that *P. gingivalis* 381 infection significantly increased secretion of MCP-1 in C57BL/6 cells by 2.0-fold at 6 hours compared to the non-infected group ($p<0.0001$, $n=4$) and by 1.7-fold compared to the DPG3-infected group ($p<0.0001$, $n=4$) (Figure 2a). As shown in Figure 2b, at 24 hours the magnitude of the increase was lower, 1.3-fold ($p=0.0026$) and 1.2-fold ($p=0.0096$) compared to control and DPG3-infected, respectively ($n=3$). Infection with the non-invasive fimbriae-deficient mutant DPG3 caused a minor increase in MCP-1 levels compared to control, which reached statistical significance at 6 hours ($p=0.0010$, $n=4$), but not at 24 hours ($p=0.2816$, $n=3$).

Consistent with an important role for RAGE in the *P. gingivalis* 381-elicited MCP-1 upregulation, and as shown in Figure 2a and b, *P. gingivalis* 381 infection did not increase MCP-1 levels in MAEC from RAGE^{-/-} mice at either time point tested ($p=0.5292$ and $p=0.0852$ compared to non-infected at 6 and 24 hours, respectively, $n=4$). DPG3 infection had no effect as well.

In addition to studies using RAGE^{-/-} MAEC, the effects of RAGE blockade on MCP-1 production after *P. gingivalis* 381 infection were tested in HAEC using anti-RAGE IgG. RAGE blockade in these experiments significantly suppressed MCP-1 production 6 hours after *P. gingivalis* 381 infection (275 ± 26 pg/ml) compared to infected cells (371 ± 57 pg/ml); $p=0.0403$, $n=3$. The non-specific IgG control antibody had no effect (359 ± 15 pg/ml); $p=0.7130$, $n=3$.

3.3 RAGE ligand levels following infection of MAEC with *P. gingivalis*

As increased levels of RAGE ligands have been shown to drive the receptor's activation and subsequent pro-inflammatory events [13], we hypothesized that *P. gingivalis* infection generates RAGE ligands. We first focused on AGEs and measured these species in MAEC supernatants. The effect of infection on AGE production depended on the status of RAGE expression in the endothelial cells (two-way ANOVA $p<0.0001$), but did not depend on the time point (two-way ANOVA $p=0.2110$ for C57BL/6 and $p=0.2406$ for RAGE^{-/-} cells).

Specifically, infection of C57BL/6 MAEC with *P. gingivalis* 381 significantly increased AGE-immunoreactive epitopes by 2.4-fold at 6 hours (Figure 3a) and by 3.3-fold at 24 hours (Figure 3b) after infection compared to the non-infected group ($p=0.0003$ and $p=0.0041$, respectively, $n=3$), or compared to the DPG3-infected group ($p=0.0003$ at 6 hours and $p=0.0046$ at 24 hours, $n=3$). Infection with DPG3 had no effect at either time point. In MAEC from RAGE $^{-/-}$ mice infection with *P. gingivalis* did not affect AGE production at either time point. Thus, these data suggest that *P. gingivalis* 381 elicits AGE generation selectively in wild-type, but not in RAGE $^{-/-}$ MAEC.

Next, we tested the induction of non-AGE RAGE ligands in HAEC infected with *P. gingivalis*. Immunoblot analysis of cellular lysates of *P. gingivalis* 381-infected HAEC revealed a decreased HMGB1 expression compared to the non-infected control or DPG3-infected HAEC, with the greatest difference observed 6 hours after infection (data not shown). This finding suggests an enhanced cellular release of this proinflammatory RAGE ligand; however, we were unable to detect measurable levels of HMGB1 in supernatant samples by ELISA or immunoblotting, even after 20-fold concentration using ultrafiltration centrifugal devices (iCON concentrator, Thermo Scientific). Similarly, S100B was not detectable by ELISA (BioVendor, Candler, NC) in supernatants of *P. gingivalis* 381-infected HAECs.

3.4 Effect of AGE inhibition and antioxidant treatment on infection-elicited MCP-1 production in primary HAEC

The unaffected MCP-1 production following infection in RAGE $^{-/-}$ MAEC suggested that the receptor is essential in mediating this proatherogenic response elicited by *P. gingivalis*. *P. gingivalis* also increased levels of AGEs in C57BL/6 MAEC, which can trigger RAGE inflammatory signaling. To directly assess the contribution of AGE ligands in this setting, we specifically blocked these species in HAEC using the AGE inhibitor aminoguanidine or anti-AGE IgG. As shown in Figure 4, both blocking agents significantly suppressed MCP-1 production by HAEC 6 hours following infection with *P. gingivalis* 381 ($p=0.0111$ and $p=0.0039$, respectively, $n=4$ for both), while non-specific IgG control had no effect ($p=0.9251$, $n=3$). Further, and since ROS generation has been previously reported following infection of ECs with *P. gingivalis* 381 [20,21], we also assessed the effect of antioxidant treatment in this setting. As shown in Figure 4, both NAC (a well-characterized antioxidant) and DPI (a NADPH oxidase inhibitor) significantly suppressed MCP-1 levels in HAEC following *P. gingivalis* 381 infection compared to infection in the absence of inhibitors ($p=0.0002$ and $p=0.0006$, respectively, $n=3$ for both). These data provide direct evidence linking *P. gingivalis* 381-elicited MCP-1 upregulation to ROS and AGE generation in ECs.

3.5 RAGE gene and protein expression in HAEC upon infection with *P. gingivalis* and following AGE and ROS inhibition

Finally, we assessed the role of ROS and AGE generation upstream, focusing on RAGE expression. First, we found that in HAEC, infection with *P. gingivalis* 381 increased RAGE transcript by 2.5 fold at 2 hours (Figure 5a) and RAGE protein by 2.0-fold at 6 hours (Figure 5b) as compared to non-infected cells ($p=0.0077$, $n=4$ and $p=0.0007$, $n=3$, respectively). Infection with the mutant strain DPG3 had no effect on RAGE mRNA expression ($p=0.3804$, $n=4$) and, in contrast to our finding with RAGE protein at 6 hours in MAEC, it also had no effect on RAGE protein level ($p=0.3301$, $n=3$). Interestingly, infection of HAEC with DPG3 did not affect MCP-1 mRNA and protein expression either ($p=0.1549$ and $p=0.5858$, $n=3$ for both). Consistent with a role for AGE and ROS generation in RAGE upregulation following *P. gingivalis* 381 infection, pretreatment with AG, NAC or DPI significantly suppressed RAGE mRNA (Figure 5c, $p=0.0017$, $p=0.0038$ and $p=0.0103$, respectively, $n=4$ for all) and protein expression (Figure 5d, $p<0.0001$ and $n=3$ for all) compared to infection in the absence of

inhibitors. These data demonstrate an important role for oxidative stress and an AGE-dependent activation of RAGE following HAEC infection with *P. gingivalis* 381.

4. Discussion

Our findings provide first time evidence that the AGE-RAGE axis amplifies infection-mediated proinflammatory and proatherogenic responses in vascular ECs. First, primary aortic ECs isolated from RAGE^{-/-} mice and infected with *P. gingivalis* 381 showed a significantly suppressed release of proatherogenic MCP-1 and AGEs compared to ECs isolated from C57BL/6 mice. Further, anti-oxidant treatment and blockade of AGE ligands, which have been shown to drive the receptor's activation and subsequent pro-inflammatory events in other settings, suppressed RAGE and MCP-1 expression in primary human aortic ECs following infection.

The major fimbriae of *P. gingivalis*, required for the pathogen's adhesion to/invasion of host cells and for virulence, appear to play a role in its potential to promote EC activation and vascular inflammation [6,10]. Indeed, in the present study, although the mutant strain lacking major fimbriae increased RAGE expression in wild-type MAEC 6 hours post infection, it did not have an effect 24 hours post infection and did not affect levels of AGE ligands at either time point. Interestingly, RAGE expression 6 hours following DPG3 infection remained unaffected in human cells. Nevertheless, it is possible that DPG3 has an effect on RAGE expression in certain cell types; its lipopolysaccharide is not distinguishable from that of the wild type strain and it also possesses all other bacterial antigens that may confer proinflammatory effects. However, the trigger to increased RAGE levels with DPG3 appears to be different than the trigger that follows infection with *P. gingivalis* 381, and this suggests to us that the rapid production of AGEs we observed following infection with *P. gingivalis* 381, but not with the mutant bacteria, may play a major role here. To determine if the ability of *P. gingivalis* 381 to invade MAEC was affected by RAGE deletion, recovery of viable microorganisms from antibiotic-treated C57BL/6 and RAGE^{-/-} MAEC was assessed using antibiotic protection assays, as previously described [10]. Mean counts of invading bacteria derived from these experiments were similar in cells isolated from C57BL/6 and in those isolated from RAGE^{-/-} mice (data not shown).

The production of pro-inflammatory cytokines/chemokines by ECs marks an important initial step in atherogenesis and previous studies have shown that HAEC produce increased chemokine levels upon infection with *P. gingivalis* [10,22]. Our present data demonstrate that infection of wild-type MAEC or HAEC with *P. gingivalis* 381 leads to an elevated production of MCP-1, a key mediator of monocyte trafficking and T lymphocyte chemoattraction. Interestingly, infection of RAGE^{-/-} MAEC under the same conditions did not elevate levels of this chemokine, suggesting that RAGE is an important mediator of this proatherogenic response.

AGEs, important proinflammatory RAGE ligands, have been shown to play a significant role in atherogenesis [23]. Our data from C57BL/6 ECs suggest that infection with *P. gingivalis* 381 causes cellular stress, possibly through ROS generation as previously suggested [20,21], resulting in enhanced generation of AGEs; this is abrogated in MAEC from mice lacking the receptor. Although intriguing, this finding parallels the suppression of AGE levels previously reported following RAGE blockade in hyperglycemic environments [14,24]. AGE interaction with endothelial RAGE can lead to further induction of intracellular ROS, activation of NF- κ B and subsequent transcriptional activation of proinflammatory genes [25]. Based on our present antioxidant treatment data, oxidative stress following infection of ECs with *P. gingivalis* appears to be primarily generated by NADPH oxidase and to be an early event that is essential to both RAGE and downstream MCP-1 expression.

Taken together, the present findings suggest an important role for the RAGE axis in the setting of infection-mediated vascular stress. It is well established that toll-like receptor (TLR) 2 plays an important role in the innate immune response mediated by *P. gingivalis* [26]. However, considering the complex nature and multifactorial etiology of atherosclerosis and periodontitis, our study offers novel insight into the interrelation of these two conditions. Importantly, it has been demonstrated that ligation of RAGE leads to persistent inflammation that is not protective, as seen in TLR-mediated pathogen clearance [27]. RAGE is indeed less effective than other multi-ligand pattern recognition receptors in mediating disposal of bound AGEs via degradation, but much more effective in soliciting downstream signaling and causing cellular perturbation and inflammation [27].

In conclusion, novel results of the present study are the first demonstration that levels of RAGE and AGE epitopes are enhanced in vascular endothelial cells following infection with *P. gingivalis* 381 and that blockade of the AGE-RAGE axis through inhibition of AGEs and ROS can exert beneficial effects in this cell type. These findings contribute to a better understanding of possible mechanisms involved in the link between periodontal infection and vascular disease.

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Figure 1. RAGE levels by immunoblotting in MAEC isolated from C57BL/6 mice
P. gingivalis 381 (Pg 381) significantly increased RAGE expression in C57BL/6 MAEC 6 hours after infection (a) compared to the non-infected cells (NI) ($p=0.0122$, $n=3$). At 24 hours (b) the magnitude of the increase was smaller ($p=0.0128$, $n=3$). Infection with the fimbriae-deficient mutant DPG3 also caused a significant increase in RAGE expression compared to NI at 6 hours ($p=0.0108$, $n=3$), but had no effect at 24 hours ($p=0.7634$, $n=3$)

Figure 2. MCP-1 production by ELISA in MAEC isolated from C57BL/6 or RAGE^{-/-} mice
 Infection of C57BL/6 MAEC with *P. gingivalis* 381 (Pg 381) significantly increased secretion of MCP-1 6 hours after infection (a) compared to non-infected cells (NI) or DPG3-infected cells ($p < 0.0001$ for both, $n = 4$). At 24 hours (b) the magnitude of the increase in C57BL/6 MAEC was smaller ($p = 0.0026$ and $p = 0.0096$ compared to NI or DPG3-infected cells, respectively, $n = 3$). DPG3 infection caused a minor MCP-1 increase in C57BL/6 MAEC compared to NI which was statistically significant at 6 hours ($p = 0.0010$, $n = 4$), but not at 24 hours ($p = 0.2816$, $n = 3$). Consistent with an important role for RAGE in this setting, infection with either strain did not affect MCP-1 production in RAGE^{-/-} MAEC.

Figure 3. AGE production by ELISA in MAEC isolated from C57BL/6 or RAGE^{-/-} mice
 (a) Infection of C57BL/6 MAEC with *P. gingivalis* 381 (Pg 381) significantly increased AGE epitopes compared to non-infected (NI) and to DPG3-infected cells at 6 hours ($p=0.0003$ for both, $n=3$). In RAGE^{-/-} MAEC *P. gingivalis* infection did not affect AGE production. (b) Similarly, 24 hours following infection of C57BL/6 MAEC with Pg 381, AGE epitopes were significantly increased compared to NI and to DPG3-infected cells ($p=0.0041$ and $p=0.0046$, respectively, $n=3$). Again, infection had no effect on AGE production in RAGE^{-/-} MAEC.

Figure 4. Effect of AGE blockade and antioxidant treatment on infection-elicited MCP-1 production in HAEC

To directly assess the effect of AGE ligands on *P. gingivalis* 381-elicited MCP-1 upregulation, we specifically blocked these species using the AGE inhibitor aminoguanidine (AG, 200 μ mol/L) or anti-AGE IgG (15 μ g/ml). Both blocking agents significantly suppressed MCP-1 production in HAEC 6 hours following infection with *P. gingivalis* 381 (Pg 381); $p=0.0111$ and $p=0.0039$, respectively, $n=4$ for both. Non-specific IgG antibody (15 μ g/ml) used as control had no effect ($p=0.9251$, $n=3$). Further, antioxidant treatment using *N*-acetyl-L-cysteine (NAC, 10 mmol/L), or diphenylene iodonium (DPI, 30 μ mol/L) also significantly suppressed MCP-1 production; $p=0.0002$ and $p=0.0006$, respectively, $n=3$ for both.

Figure 5. RAGE mRNA and protein levels, by Real Time PCR and immunoblotting, in HAEC (a) *P. gingivalis* 381 (Pg 381) significantly increased RAGE mRNA in HAEC 2 hours after infection compared to the non-infected cells (NI) (0.0077, n=4), while infection with the fimbriae-deficient mutant DPG3 had no such effect (p=0.3804, n=4). The results were similar on the protein level (b); p=0.0007 and p=0.3301, respectively, n=3 for all. (c) Consistent with a role for AGE and ROS generation in RAGE upregulation following *P. gingivalis* 381 infection, pretreatment with aminoguanidine (AG, 200 μmol/L), *N*-acetyl-L-cysteine (NAC, 10 mmol/L), or diphenylene iodonium (DPI, 30 μmol/L) significantly suppressed RAGE mRNA (p= 0.0017, p=0.0038 and p=0.0103, respectively, n=4 for all). This effect was demonstrated on the protein level as well (d); p<0.0001 and n=3 for all.