

Advanced glycation endproducts mediate pro-inflammatory actions in human gestational tissues via nuclear factor- κ B and extracellular signal-regulated kinase 1/2

Martha Lappas¹, Michael Permezel¹ and Gregory E Rice^{1,2}

¹Department of Obstetrics and Gynaecology, Mercy Hospital for Women, University of Melbourne, Level 4/163 Studley Road, Heidelberg, Victoria 3084, Australia

²Translational Proteomics, Baker Medical Research Institute, Baker Heart Research Institute, Melbourne, Victoria 3004, Australia

(Requests for offprints should be addressed to M Lappas; Email: mlappas@unimelb.edu.au)

Abstract

Processes of human labour include increased oxidative stress, formation of inflammatory mediators (e.g. cytokines) and uterotonic phospholipid metabolites (e.g. prostaglandins). In non-gestational tissues, advanced glycation endproducts (AGE) induce the expression of pro-inflammatory molecules through mitogen-activated protein kinase and nuclear factor κ B (NF- κ B)-dependent pathways. Thus, the aim of this study was to investigate the effects of AGE on 8-isoprostane (a marker of oxidative stress), pro-inflammatory cytokine and prostaglandin release in human gestational tissues, and to define the signalling pathways involved. Human placenta and gestational membranes (amnion and choriodecidua combined; $n=5$) were incubated in the absence or presence of AGE-BSA (0.25, 0.5, 1 and 2 mg/ml) for 18 h. AGE

significantly increased *in vitro* release of tumour necrosis factor- α , interleukin (IL)-1 β , IL-6, IL-8, prostaglandin (PG)E₂, PGF_{2 α} and 8-isoprostane from human placenta and gestational membranes. This was associated with a concomitant increase in NF- κ B p65 activation and ERK 1/2 phosphorylation. AGE-stimulated 8-isoprostane, cytokine and prostaglandin production was significantly suppressed by the ERK 1/2 inhibitor U0126 and the NF- κ B inhibitor BAY 11-7082. In conclusion, AGE mediates inflammatory actions in human gestational tissues. Protein kinases and the NF- κ B pathway play an essential role in AGE signalling in human gestational tissues.

Journal of Endocrinology (2007) **193**, 269–277

Introduction

Increased intrauterine cytokine and prostaglandin production, which occurs modestly with term labour, is a characterising feature of preterm labour, particularly in the presence of intrauterine infection (Kniss 1999, Rice 2001, Bowen *et al.* 2002, Lappas and Rice 2004). Bacterial colonisation and/or inflammation of the choriodecidual interface induces production of pro-inflammatory cytokines that, in turn, lead to neutrophil activation and the synthesis and release of uterotonins such as prostaglandins, which cause uterine contractions, and metalloproteinases, that weaken fetal membranes and remodel cervical collagen (Rice 2001, Bowen *et al.* 2002). Recent evidence suggests that advanced glycation endproducts (AGE), which accumulate in diverse biological settings such as diabetes, aging and inflammation, exhibit immunomodulatory functions and therefore may be involved in many normal and abnormal physiological processes (Singh *et al.* 2001, Ramasamy *et al.* 2005). Both AGE and receptor for AGE (RAGE) have recently been identified within the human intrauterine environment (Cooke *et al.*

2003, Konishi *et al.* 2004, Tsukahara *et al.* 2004, Chekir *et al.* 2006). Specifically, RAGE expression has recently been identified in human myometrium (Cooke *et al.* 2003), first trimester human chorionic villi (Tsukahara *et al.* 2004) and human term placenta (Chekir *et al.* 2006). Furthermore, two known AGEs, pentosidine and *N*-carboxymethyllysine protein have been detected in umbilical cord blood (Konishi *et al.* 2004) and human term placenta (Chekir *et al.* 2006) respectively. These data support the hypothesis that the AGE–RAGE axis may play a role in the processes of human labour and delivery, mainly cytokine and prostaglandin production.

The formation of AGE occurs through the non-enzymatic reactions between sugars and the free amino groups on proteins, lipids and nucleic acids (Maillard 1912, John & Lamb 1993). Under physiological conditions, most AGE-modified proteins in plasma undergo rapid plasma clearance (Takata *et al.* 1988); however, the formation of AGE proteins beyond physiological levels or impairment of the AGE elimination system potentially results in the accumulation of AGE in tissues (Smith *et al.* 1994, Nishino *et al.* 1995, Anderson *et al.*

1999). As AGE levels rise, interruption of normal function occurs via receptor-independent and/or -dependent pathways. In receptor-dependent pathways, AGE-modified proteins bind inflammatory cells by interaction with specific cell surface molecules (e.g. macrophage scavenger receptor type II, CD36 and RAGE). RAGE, which is the best studied pro-inflammatory AGE receptor, is a multiligand type I transmembrane glycoprotein belonging to the immunoglobulin superfamily (Neeper *et al.* 1992). The RAGE ligands include AGE, amyloid- β peptide, amphoterin and several members of the S100 protein superfamily (reviewed in Bucciarelli *et al.* 2002, Chavakis *et al.* 2004, Bierhaus *et al.* 2005). RAGE has potential involvement in several pathological processes including diabetes, inflammation, ageing, neurodegenerative disorders, Alzheimer's disease and tumours (reviewed in Bucciarelli *et al.* 2002, Chavakis *et al.* 2004, Bierhaus *et al.* 2005, Ramasamy *et al.* 2005). Ligand-receptor interaction, through the generation of reactive oxygen species (ROS), induces sustained post-receptor signalling including activation of p21ras, the mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK)-1 and -2, p38 MAP kinase, and downstream consequences such as activation of nuclear factor κ B (NF- κ B), which subsequently induces the expression of adhesion molecules, cytokines and chemokines (reviewed in Bucciarelli *et al.* 2002, Chavakis *et al.* 2004, Bierhaus *et al.* 2005, Ramasamy *et al.* 2005). We, and other researchers, have previously demonstrated that in human gestational tissues, ERK 1/2 and NF- κ B regulate pro-inflammatory proteins including cytokines, prostaglandins and proteases (Lappas *et al.* 2003, 2004, 2005, Jung *et al.* 2005, Sooranna *et al.* 2005), all of which are known to be increased in human labour.

Whether or not AGE regulates phospholipid metabolism and inflammatory cytokines in human gestational tissues has yet to be established. The hypothesis tested is that AGE induces the release of labour-promoting mediators in human placenta and gestational membranes *in vitro*. A well characterised *ex situ* human tissue explant incubation model (Lappas *et al.* 2003, 2004, 2005) will be used to investigate the effects of AGE-BSA on cytokine and prostaglandin release from human placenta and gestational membranes. In addition, the role of the signalling proteins ERK 1/2 and NF- κ B involved in this regulation will also be investigated.

Materials and Methods

Reagents

All chemicals were purchased from BDH Chemicals Australia (Melbourne, Victoria, Australia) unless otherwise stated. RPMI 1640 (phenol red free) was obtained from Gibco Laboratories (Grand Island, NY, USA). AGE-BSA and non-glycated BSA, BSA (RIA grade), dithiothreitol, EDTA, leupeptin, β -NADH (disodium salt), 3,3',5,5'-tetramethylbenzidine and pyruvic acid (dimer-free) were supplied by Sigma. Pefabloc SC was purchased

from RocheMolecular Biochemicals. (E)-3-[4-methylphenylsulphonyl]-2-propenenitrile (BAY 11-7082) was purchased from Sapphire Bioscience (Crows Nest, NSW, Australia). U0126 was purchased from Tocris (Ellisville, MO, USA). The TNF- α , IL-1 β , IL-6 and IL-8 ELISA kits were supplied by Biosource International (Camarillo, CA, USA). The PGE₂, PGF_{2 α} and 8-isoprostane EIA kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Criterion 10% Tris-HCl gels and kaleidoscope pre-stained molecular weight markers were purchased from Bio-Rad. Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane and Hyperfilm ECL were supplied by Amersham Biosciences.

Tissue collection and preparation

Human placentae and attached gestational membranes were obtained (with Institutional Research and Ethics Committee approval) from a total of ten women ($n=5$ women for Part 1 and $n=5$ women for Part 2), who delivered healthy, singleton infants at term (>37 weeks gestation) undergoing elective caesarean section (indications for caesarean section were breech presentation and/or previous caesarean section). Tissues were obtained within 10 min of delivery and dissected fragments were placed in ice-cold RPMI. A placental lobule (cotyledon) was removed from the central region of the placenta. The basal plate and chorionic surface were removed from the cotyledon, and the villous tissue was obtained from the middle cross-section. Placental tissue was bluntly dissected to remove visible connective tissue and calcium deposits. Fetal membranes (amnion and choriondecidua combined) were obtained 2 cm away from the pre-placental edge, and explants were prepared by sharp dissection of 2.5 cm². Tissue fragments were placed in RPMI at 37 °C in a humidified atmosphere of 8% O₂ and 5% CO₂ for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (200 mg wet weight/well). The explants were incubated, in triplicate, in 2 ml RPMI containing penicillin G (100 U/ml) and streptomycin (100 μ g/ml). All explant incubations were performed for 18 h under 8% O₂ and 5% CO₂.

Explants ($n=4-5$) were incubated in the absence or presence of increasing concentrations of AGE-BSA (0.25, 0.5, 1 and/or 2 mg/ml). The AGE-BSA concentration used was based on previously published manuscripts (for examples see Denis *et al.* 2002, Konishi *et al.* 2004, Ge *et al.* 2005), and is expressed as the BSA protein concentration added in the incubation medium. The AGE content in the preparations is 3 mol AGE per mol BSA. To further explore the relationship between AGE-BSA and the inflammatory response, various pharmacological agents were supplemented to the explant incubation medium, including 50 μ M BAY 11-7082 and 10 μ M U0126 ($n=3$). The concentrations used were based on the results of our previously published dose-response inhibition experiments (Lappas *et al.* 2005). After 18 h incubation, the medium was collected and stored at -80 °C until assayed for cytokine and prostaglandin release

by ELISA. Tissue was collected and cytoplasmic and nuclear protein was immediately extracted (as detailed in Lappas *et al.* 2003, 2004, 2005) and stored at -80°C until assayed for ERK and NF- κ B activation by ELISA.

Validation of explant cultures and viability

To determine the effect of treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as described previously (as detailed in Lappas *et al.* 2003, 2004, 2005). LDH release was investigated over the 18 h time course of tissue explants. Explants were incubated in either medium alone or medium containing 1 mg/ml AGE-BSA. Neither *in vitro* incubation nor experimental treatment significantly affected LDH activity in the incubation medium (data not shown). These data indicate that the concentrations used in this study did not affect cell viability.

Experimental assays

After 18 h incubation, the explant incubation medium was collected and the release of leptin, TNF- α , IL-1 β , IL-6 and IL-8 was tested by sandwich ELISA, according to the manufacturer's instructions (Biosource International). The limit of detection of the TNF- α , IL-1 β , IL-6 and IL-8 assays (defined as 2 s.d. from the zero standard) were 7.2, 7.2, 2.8, 15.6 and 15.6 pg/ml respectively. The release of PGE₂ and PGF_{2 α} into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company). The limits of detection of the PGE₂ and PGF_{2 α} enzyme immuno assay (EIA) kits were 8.0 and 14.9 pg/ml respectively. The release of 8-isoprostane into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company). The limit of detection of the assay was 5 pg/ml. All data were corrected for total protein and expressed as picograms per milligram protein. The protein content of tissue explants was determined using BCA protein assay (Pierce, Rockford, IL, USA), using BSA as a reference standard, as previously described (as detailed in Lappas *et al.* 2003, 2004, 2005).

Assessment of NF- κ B p65 DNA binding activity and ERK 1/2 phosphorylation

Total and phosphorylated ERK 1/2 in extracts was assessed using a commercially available kit according to the manufacturer's instructions (Assay Designs, Ann Arbor, Michigan, USA). A Bio-Rad Benchmark Microplate Reader was used to read the sample absorbance, with data expressed as a percentage of phosphorylated/total ERK 1/2.

NF- κ B DNA binding in nuclear protein extracts was assessed using a commercially available NF- κ B p65

transcription factor ELISA according to the manufacturer's instructions (Cayman Chemical Company), where TNF- α -stimulated HeLa nuclear protein extract was used as a positive control for NF- κ B activation, and specificity of NF- κ B binding was assessed using wild-type and mutated consensus oligonucleotides. A Bio-Rad Benchmark Microplate Reader was used to read the sample absorbance, with data expressed as optical density at 450 nm.

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, MD, USA). Homogeneity of data was assessed by Bartlett's test, and when significant, data were logarithmically transformed before further analysis. The effect of experimental treatment on cytokine and prostaglandin release was analysed by M-ANOVA. The method used to discriminate among the means is Fisher's least significant difference procedure. Statistical difference was indicated by a $P < 0.05$. Data are expressed as mean \pm s.e.m.

Results

Part 1: does AGE-BSA induce an inflammatory response in human gestational tissues?

In non-gestational tissues, previous studies have shown that AGE induces pro-inflammatory protein expression. Thus, the first part of this study aimed to investigate the effect of AGE-BSA on the formation of pro-labour mediators in human gestational tissues. An initial dose-response experiment was performed to determine the working concentration of AGE-BSA. Human placenta and gestational membranes were incubated in the absence or presence of 0.25, 0.5, 1 and 2 mg/ml AGE-BSA. After 18 h incubation, the incubation medium was collected and assayed for IL-6 release by ELISA. Basal IL-6 release from both placenta and fetal membranes was significantly increased in the presence of all concentrations of AGE-BSA (Fig. 1a), with 0.5 and 1 mg/ml AGE-BSA eliciting the greatest IL-6 response. Furthermore, there was a bi-phasic response, with the highest concentration of 2 mg/ml AGE-BSA eliciting a significantly lower release of IL-6 when compared with the lower concentrations of 0.25, 0.5 and 1 g/ml AGE-BSA (Fig. 1a). Non-glycated BSA (at 1 mg/ml), used as a control, had no significant effect on basal IL-6 release from human placenta and gestational membranes (Fig. 1b). As the greatest cytokine response was observed in the presence of 0.5 and 1 mg/ml AGE-BSA for both placenta and fetal membranes, all subsequent experiments for Part 1 were performed in the presence of these concentrations.

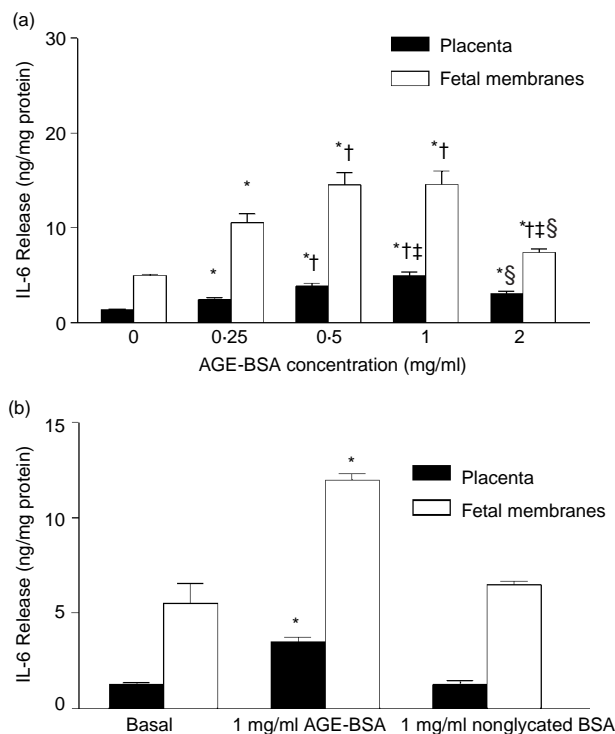
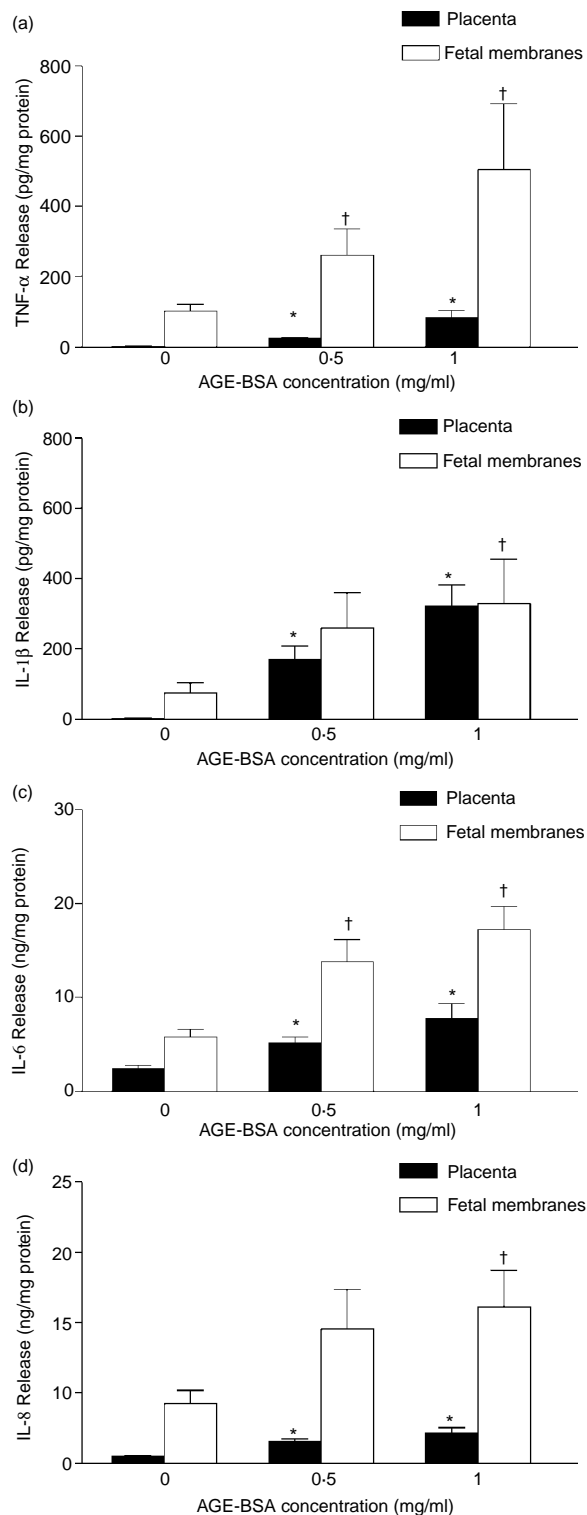


Figure 1 (a) AGE-BSA dose-response: effect of AGE-BSA and nonglycated BSA on IL-6 release from human placenta and gestational membranes ($n=3$). Tissues were incubated for 18 h in the absence or presence of increasing concentrations of AGE-BSA (0.25, 0.5, 1 and 2 mg/ml). (b) Effect of non-glycated BSA on IL-6 release from human placenta and gestational membranes ($n=3$). Tissues were incubated for 18 h in the absence or presence of 1 mg/ml AGE-BSA or 1 mg/ml nonglycated BSA. Each bar represents the mean \pm S.E.M. * $P<0.05$ versus basal IL-6 release; † $P<0.05$ versus 0.25 mg/ml AGE-stimulated IL-6 release; ‡ $P<0.05$ versus 0.5 mg/ml AGE-stimulated IL-6 release; § $P<0.05$ versus 1 mg/ml AGE-stimulated IL-6 release.

Effect of AGE-BSA on the release of pro-inflammatory cytokines Human placenta and gestational membranes ($n=5$) were incubated in the absence or presence of 0.5 and 1 mg/ml AGE-BSA. After 18 h incubation, the incubation medium was collected and assayed for cytokine release. The release of TNF- α (Fig. 2a), IL-1 β (Fig. 2b), IL-6 (Fig. 2c) and IL-8 (Fig. 2d) from human placenta was significantly increased by both 0.5 and 1 mg/ml AGE-BSA. In human gestational membranes, both 0.5 and 1 mg/ml AGE-BSA significantly increased TNF- α and IL-6 release, however, only 1 mg/ml AGE-BSA significantly increased IL-1 β and IL-8 release from human gestational membranes (Fig. 2a–d). There was no significant effect of non-glycated BSA (at 1 mg/ml) on the release of IL-1 β , IL-6, IL-8 and TNF- α from placenta or gestational membranes (data not shown).

Effect of AGE-BSA on the release of prostaglandins Human placenta and gestational membranes ($n=5$) were incubated in the absence or presence of 0.5 and 1 mg/ml



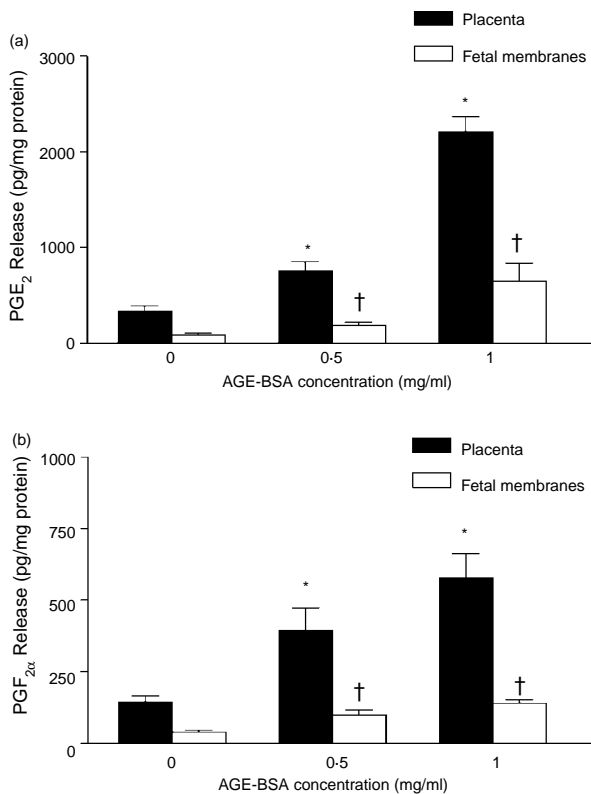


Figure 3 Effect of AGE-BSA on (a) PGE₂ and (b) PGF_{2α} release from human placenta and gestational membranes ($n=5$). Tissues were incubated for 18 h in the absence or presence of 0.5 and 1 mg/ml AGE-BSA. Each bar represents the mean \pm S.E.M. * $P<0.05$ versus basal prostaglandin release from placenta; † $P<0.05$ versus basal prostaglandin release from gestational membranes.

AGE-BSA. After 18 h incubation, the incubation medium was collected and assayed for prostaglandin release. PGE₂ (Fig. 3a) and PGF_{2α} (Fig. 3b) release was significantly amplified in placenta and gestational membranes by treatment with both 0.5 and 1 mg/ml AGE-BSA. There was no significant effect of non-glycated BSA (at 1 mg/ml) on PGE₂ or PGF_{2α} release from both human placenta and gestational membranes (data not shown).

Effect of AGE-BSA on the release of 8-isoprostane

Previous studies have shown that AGE induces oxidative stress (Denis *et al.* 2002), resulting in enhanced inflammation. Isoprostanes are prostaglandin-like products derived from free radical-catalysed non-enzymatic oxidation of arachidonic acid (Morrow *et al.* 1990), and are considered to be an accurate and reliable marker of oxidative stress and

Figure 2 Effect of AGE-BSA on (a) TNF- α , (b) IL-1 β , (c) IL-6 and (d) IL-8 release from human placenta and gestational membranes ($n=5$). Tissues were incubated for 18 h in the absence or presence of 0.5 and 1 mg/ml AGE-BSA. Each bar represents the mean \pm S.E.M. * $P<0.05$ versus basal cytokine release from placenta; † $P<0.05$ versus basal cytokine release from gestational membranes.

endogenous lipid peroxidation (Awad *et al.* 1996). We therefore examined the effect of AGE on the production of 8-isoprostane from human placenta and gestational membranes. The tissues ($n=5$) were incubated in the absence or presence of 0.5 and 1 mg/ml AGE-BSA, and after 18 h incubation, the incubation medium was collected and assayed for 8-isoprostane release by EIA. AGE-BSA at both 0.5 and 1 mg/ml significantly increased the release of 8-isoprostane from human placenta and gestational membranes (Fig. 4).

Part 2: intracellular signalling pathways involved in the pro-inflammatory actions of AGE-BSA

Having established that AGE-BSA stimulates the release of pro-inflammatory mediators from human gestational tissues, the next aim was to determine the intracellular signalling pathways involved. Therefore, ELISA was performed to determine the effect of 1 mg/ml AGE-BSA on ERK 1/2 phosphorylation and NF- κ B activation. Incubation of placenta and gestational membranes with 1 mg/ml resulted in a significant increase in NF- κ B p65 DNA-binding activity (Fig. 5a) and ERK 1/2 phosphorylation (Fig. 5b). Furthermore, NF- κ B and ERK 1/2 activation was significantly suppressed in the presence of the specific inhibitors BAY 11-7082 (at 50 μ M) and U0126 (at 10 μ M) respectively (Fig. 5b).

Having established that AGE-BSA activated NF- κ B and induces the phosphorylation of ERK 1/2 in human placenta and gestational membranes, we then used the NF- κ B inhibitor BAY 11-7082 and the ERK 1/2 inhibitor U0126 to determine the possible signal transduction pathways involved in AGE-BSA-induced pro-inflammatory mediator release from human intrauterine tissues. After 18 h incubation, the incubation medium was collected and assayed for cytokine, prostaglandin and 8-isoprostane release. Treatment of tissue explants with 10 μ M U0126 and 50 μ M BAY 11-7082 significantly inhibited

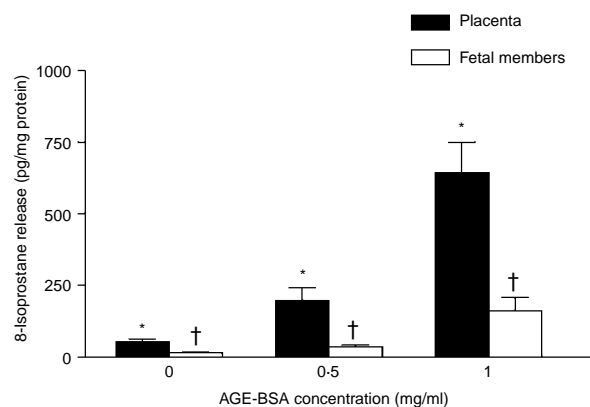


Figure 4 Effect of AGE-BSA on 8-isoprostane release from human placenta and gestational membranes ($n=5$). Tissues were incubated for 18 h in the absence or presence of 0.5 and 1 mg/ml AGE-BSA. Each bar represents the mean \pm S.E.M. * $P<0.05$ versus basal 8-isoprostane release from placenta; † $P<0.05$ versus basal 8-isoprostane release from gestational membranes.

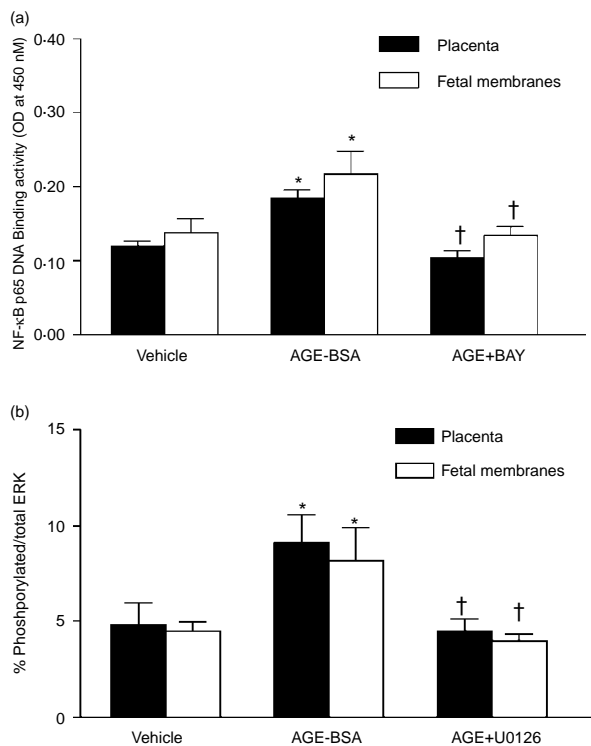


Figure 5 Effect of AGE-BSA on (a) NF- κ B activation and (b) ERK 1/2 phosphorylation in the absence or presence of BAY 11-7082 (BAY) or U0126 from human placenta and gestational membranes ($n=3$). Tissues were incubated for 18 h in the presence of 1 mg/ml AGE-BSA with or without 50 μ M BAY 11-7082 or 10 μ M U0126. Each bar represents the mean \pm s.e.m. * $P<0.05$ versus basal NF- κ B or ERK 1/2 activation; † $P<0.05$ versus AGE-BSA stimulated NF- κ B or ERK 1/2 activation.

AGE-BSA-induced release of the cytokines TNF- α , IL-1 β , IL-6 and IL-8 (Fig. 6a–d), the prostaglandins PGE₂ and PGF_{2 α} (Fig. 7a and b) and 8-isoprostane (Fig. 8) from both human placenta and gestational membranes. In order to negate any non-specific effects of U0126 and BAY 11-7082, leptin release was analysed. There was no effect of either 10 μ M U0126 and 50 μ M BAY 11-7082 on leptin release from placenta and fetal membranes (data not shown).

Discussion

In this study, we employed a well-characterised *in vitro* human tissue explant system to examine the potential inflammatory effects of AGE-BSA in human intrauterine tissues. Our data show that AGE-BSA, but not non-glycated BSA, has pro-inflammatory actions in human gestational tissues. Specifically, AGE-BSA stimulated the release of the pro-inflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α and prostaglandins PGE₂ and PGF_{2 α} . Furthermore, AGE-BSA increased the release of 8-isoprostane, a marker of oxidative stress. These pro-inflammatory actions of AGE-BSA were

elicited through a number of intracellular signalling pathways, namely ERK 1/2 and NF- κ B.

AGE can induce the expression and activation of a number of transcription factors including NF- κ B and MAP kinase (Smith *et al.* 1994, Nishino *et al.* 1995, Anderson *et al.* 1999), both of which have been implicated in the processes of human labour and delivery (Lappas *et al.* 2003, 2004, 2005, Jung *et al.* 2005, Sooranna *et al.* 2005). Similarly in this study, we have demonstrated that AGE-BSA induces an increase in NF- κ B p65 DNA binding activity and ERK 1/2 phosphorylation in human gestational tissues. This effect may be both direct (through interaction with RAGE) and/or indirect, via generation of free oxygen radicals. This leads to the production of pro-inflammatory cytokines, growth factors, adhesion molecules and chemokines (Schmidt *et al.* 1994, Hofmann *et al.* 1999, Fiuza *et al.* 2003, Shanmugan *et al.* 2006) that have been implicated in the processes of human labour and delivery (Kniss 1999, Rice 2001, Bowen *et al.* 2002, Lappas *et al.* 2004). In isolated human first-trimester trophoblasts, AGE stimulates secretion of chemokines such as macrophage inflammatory protein (MIP)-1 α and MIP-1 β , induces apoptosis, and suppresses the secretion of human chorionic gonadotrophin, an effect that could be suppressed by inhibitors of NO synthases or the NF- κ B pathway (Konishi *et al.* 2004), thus suggesting that reactive nitrogen species as well as ROS contribute to AGE-mediated actions in the human gestational tissues. Likewise, in this study, we have demonstrated that AGE induces pro-inflammatory cytokine and prostaglandin release from human gestational tissues. In addition, by arresting activation of central signalling pathways, we demonstrated that these actions of AGE were mediated by ERK 1/2 and NF- κ B.

The AGEs can increase oxidative stress by either excessive generation of ROS (dependent on RAGE), or by antioxidant depletion (independent of RAGE; Bierhaus *et al.* 1997). As a consequence, increased cellular oxidative stress leads to the activation of NF- κ B and thus promotes the upregulation of various NF- κ B-controlled genes (Lappas *et al.* 2003). As the resultant increase in ROS levels cannot be measured directly, products of oxidative stress, that is oxidative or damage, can be detected in target biomolecules. These include products of lipid peroxidation, such as 8-isoprostane. The data obtained in this study are also consistent with AGE-BSA increasing oxidative stress as evidenced by increased release of 8-isoprostane from placenta and gestational membranes. To further elucidate the mechanisms by which AGE affects cellular responses, the effects of inhibitors of ERK 1/2 and NF- κ B were assessed. All agents demonstrated antioxidative capacity, almost completely preventing the AGE-BSA-dependent induction of 8-isoprostane release from both placenta and gestational membranes.

Prostaglandins and pro-inflammatory cytokines, which are produced within the intrauterine environment, participate in the processes of human labour and delivery, specifically the regulation of myometrial contractility, cervical ripening and rupture of membranes (reviewed in Kniss 1999, Rice 2001,

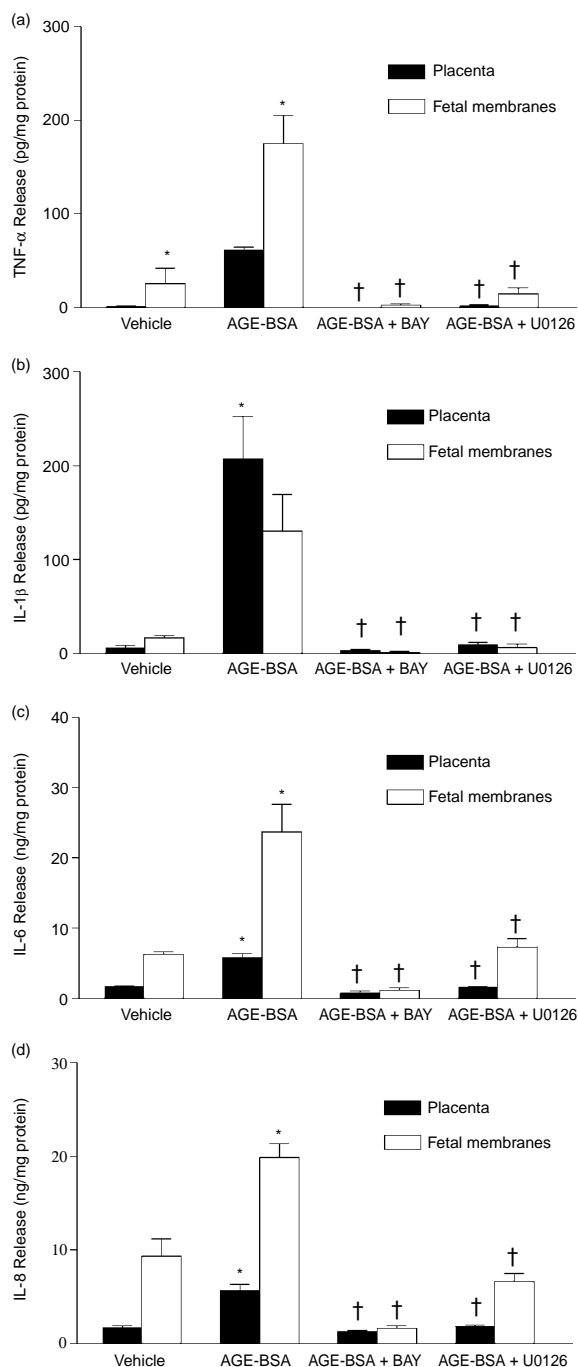


Figure 6 Effect of BAY 11-7082 and U0126 on AGE-BSA-stimulated (a) IL-1 β , (b) TNF- α , (c) IL-6 and (d) IL-8 release from human placenta and gestational membranes ($n=3$). Tissues were incubated for 18 h in the presence of 1 mg/ml AGE-BSA with or without 50 μ M BAY 11-7082 or 10 μ M U0126. Each bar represents the mean \pm S.E.M. * $P < 0.05$ versus basal cytokine release; † $P < 0.05$ versus AGE-BSA stimulated cytokine release.

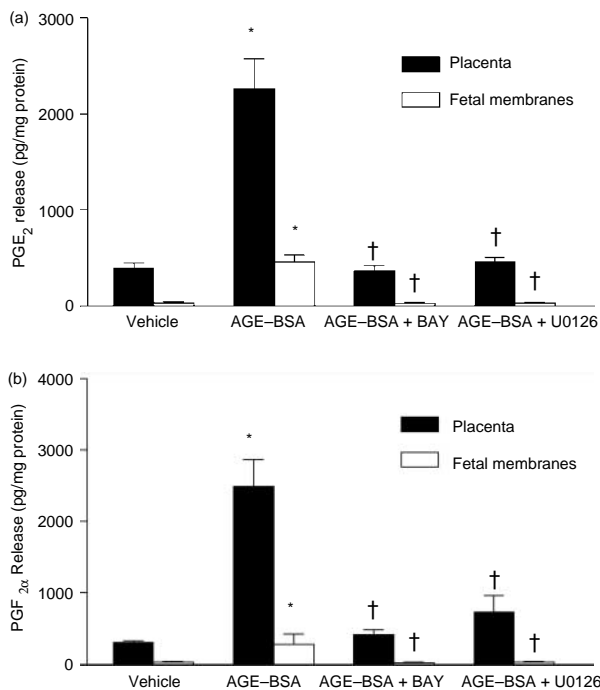


Figure 7 Effect of BAY 11-7082 and U0126 on AGE-BSA-stimulated (a) PGE₂ and (b) PGF_{2 α} release from human placenta and gestational membranes ($n=3$). Tissues were incubated for 18 h in the presence of 1 mg/ml AGE-BSA with or without 50 μ M BAY 11-7082 or 10 μ M U0126. Each bar represents the mean \pm S.E.M. * $P < 0.05$ versus basal prostaglandin release; † $P < 0.05$ versus AGE-BSA stimulated prostaglandin release.

Bowen *et al.* 2002, Lappas *et al.* 2004). Experimental data from cell culture, animal models and tissue studies have provided evidence that pro-inflammatory cytokines and prostaglandins accumulate in the amniotic fluid and intrauterine tissues of women at the time of labour at term, and they are elevated even further in infection-associated preterm labour. Further, oxidative stress also plays a role during

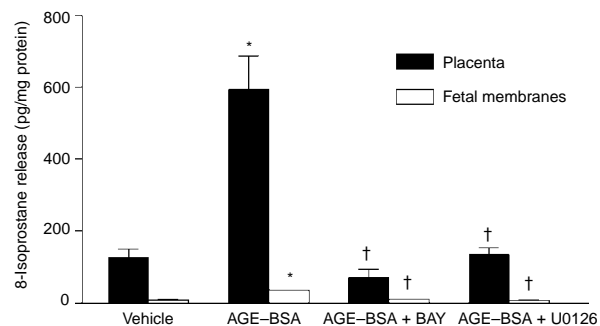


Figure 8 Effect of BAY 11-7082 and U0126 on AGE-BSA-stimulated 8-isoprostane release from human placenta and gestational membranes ($n=3$). Tissues were incubated for 18 h in the presence of 1 mg/ml AGE-BSA with or without 50 μ M BAY 11-7082 or 10 μ M U0126. Each bar represents the mean \pm S.E.M. * $P < 0.05$ versus basal 8-isoprostane release; † $P < 0.05$ versus AGE-BSA stimulated 8-isoprostane release.

normal parturition (Fainaru *et al.* 2002, Mocatta *et al.* 2004) in the initiation of preterm labour (Wall *et al.* 2002, Pressman *et al.* 2003), and in preterm, premature rupture of the membranes (Wall *et al.* 2002). Therefore, enhanced AGE and/or RAGE formation may contribute to the raised circulating levels of pro-inflammatory mediators that are evident in this state. In this study, although we have not demonstrated that the pro-inflammatory actions of AGE are mediated via RAGE, the finding that RAGE is expressed in trophoblasts in the chorionic villi of first-trimester (Konishi *et al.* 2004) and term placentas (Chekir *et al.* 2006) from healthy women suggests that RAGE may play a physiological role in the placenta, and that accumulation of AGE may interfere with the physiological role of RAGE. Given that AGE and RAGE have both been identified in the intrauterine environment (Konishi *et al.* 2004, Chekir *et al.* 2006) and that AGE increases the release of pro-inflammatory cytokines, prostaglandins and oxidative stress from human gestational tissues, the AGE-RAGE axis may play a role in the processes of human labour and delivery, particularly in preterm labour. However, further studies are required to elucidate whether or not there are also cellular changes in expression of cytokines and prostaglandins. This would provide important additional insight into the mechanisms for the AGE effects.

In this study, an *in vitro* tissue explant model system was utilised to highlight a role for AGE in the up-regulation of pro-labour mediator release from human gestational tissues, an effect that was mediated by ERK 1/2 and NF- κ B. A 'two hit' model has been proposed, where an initial AGE-RAGE interaction results in cellular activation and inflammation, followed by generation of ROS, amplified production of AGE, more inflammation and so on. In addition, the promoter region of the RAGE receptor gene contains NF- κ B binding sites (Li & Schmidt 1997), potentially producing a self-perpetuating pathway. It may therefore be feasible that elevations in the secretion of pro-inflammatory mediators from the placenta and gestational membranes in response to elevated AGE may not only be involved in normal processes of human labour at term, but may also induce the pathophysiological effects that contribute to adverse pregnancy outcomes. In support of this, RAGE is elevated in women with pre-eclampsia (Cooke *et al.* 2003), and concentrations of AGE-modified products in umbilical cord blood increase with gestation progression and in pre-eclampsia (Tsukahara *et al.* 2004). However, further studies are required on the role and regulation of AGE-RAGE in human gestational tissues to further elucidate their importance in human parturition.

Acknowledgements

Dr M Lappas was a recipient of a National Health and Medical Research Council R D Wright Fellowship (grant no. 454777). The work described in this manuscript was funded by an NHMRC project grant (number 367615). The authors gratefully acknowledge the assistance of the Clinical Research

Midwives Valerie Bryant, Margaret Cotter, Gabrielle de Bruyn and Anne Beeston, and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation. The authors would also like to gratefully acknowledge Sarah Jane Holdsworth from the Department of Obstetrics & Gynaecology, University of Melbourne, Mercy Hospital for Women for her excellent technical assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 4 February 2007

Accepted 6 February 2007

Made available online as an Accepted Preprint

14 February 2007