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Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: A role for Nrf2 in vascular protection in diabetes

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Heme oxygenase-1;
NQO1;
c-Jun terminal kinase

Abstract *Background and aims:* Hyperglycemia and diabetes are associated with increased formation of advanced glycation end products and enhanced oxidative stress, leading to the progression of diabetic vascular disease. We have investigated the mechanisms by which AGE-modified bovine albumin (AGE-BSA) induces reactive oxygen species (ROS) generation, leading to nuclear factor-erythroid 2-related factor (Nrf2) dependent induction of the antioxidant genes heme oxygenase-1 (HO-1) and NADPH:quinone oxidoreductase 1 (NQO1) in bovine aortic endothelial cells.

Methods and results: AGE-BSA (100 μg ml^{-1}, 0–24 h), but not native BSA, elicited time-dependent increases in ROS generation, Nrf2 nuclear translocation and enhanced mRNA and protein expression of HO-1 and NQO1, but not glutathione peroxidase-1. Inhibition of ROS production with the superoxide scavenger Tiron or inhibitors of flavoproteins (diphenylene iodonium) and NADPH oxidase (apocynin), but not eNOS (L-NAME) or mitochondria complex I (rotenone) abrogated HO-1 induction by AGE-BSA. Although AGE-BSA induced rapid phosphorylation of JNK and Akt, only inhibition of JNK abrogated HO-1 expression, implicating the involvement of the JNK signaling pathway in AGEs activation of Nrf2/ARE-linked antioxidant gene expression.

Abbreviations: BAEC, Bovine aortic endothelial cells; AGE, Advanced glycation end products; BSA, Bovine serum albumin; RAGE, Receptor for advanced glycation end products; HO-1, Heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; GPx-1, Glutathione peroxidase-1; Nrf2, Nuclear factor-erythroid 2-related factor; Keap1, Kelch-like *E*CH-*a*ssociated *p*rotein 1; ARE, Antioxidant response element; ROS, Reactive oxygen species; DEM, Diethylmaleate; eNOS, Endothelial nitric oxide synthase; MAPK, Mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; DPI, Diphenyl iodonium; APO, Apocynin; L-NAME, N^G-nitro-L-arginine; PI3-K, Phosphatidylinositol 3-kinase.

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Conclusion: Our findings establish that AGEs activate redox sensitive Nrf2-dependent antioxidant gene expression in bovine aortic endothelial cells, providing an adaptive endogenous defense against oxidative stress in diabetes.

Introduction

Diabetes is associated with an increased risk of microvascular complications and cardiovascular disease, with progression of the disease leading to blindness, end-stage renal failure and atherosclerosis [1]. Numerous studies have shown that hyperglycemia promotes the formation of advanced glycation end products (AGEs), which in turn affect cellular responses, survival and the progression of vascular complications in diabetes [2—4]. Non-enzymatic reaction of glucose with N-terminal amino acid residues and/or ε-amino groups of proteins forms Schiff base adducts which rearrange slowly into Amadori-modified products and the formation of AGEs, with glycated albumin the major form of circulating glycated protein *in vivo* in diabetes [4].

In endothelial cells the interaction of AGEs with the cell surface receptor for AGEs (RAGE) induces generation of reactive oxygen species (ROS), NFκB translocation and expression of pro-coagulatory and pro-inflammatory molecules such as VCAM-1 and ICAM-1 [3,4]. Under physiological conditions, RAGE is present at low levels in endothelial cells but expression is upregulated in diabetes and by AGEs themselves. Blockade of RAGE using anti-RAGE IgG or a soluble form of the extracellular domain of RAGE inhibits the development of diabetic complications and AGEs induced activation of signaling pathways [4].

ROS are produced continuously as natural by-products of the normal metabolism of oxygen and play important roles in redox signaling [5,6]. Cells have also evolved highly regulated endogenous antioxidant defense systems to counteract an overproduction of ROS in vascular diseases. The redox sensitive transcription factor nuclear factor-erythroid 2-related factor (Nrf2) forms heterodimers with small Maf proteins which bind to the antioxidant/electrophile response element (ARE/EpRE) in the promoter region of antioxidant (heme oxygenase-1) and phase II detoxifying (NAD(P)H:quinone oxidoreductase 1) enzymes to upregulate gene expression [7]. Under physiological conditions and low oxidative stress, Nrf2 is retained in the cytosol via the actin binding protein Kelch-like ECH-associated protein (Keap1) which negatively regulates Nrf2 by targeting it for ubiquitination and proteasomal degradation [8]. Oxidative and electrophilic stress induce alterations in the Nrf2-Keap1 complex, preventing proteasomal degradation and enhance Nrf2/ARE/EpRE-linked gene transcription [9].

Previous studies have reported that AGEs activate NFκB in mononuclear cells from type 1 diabetic patients [10] and the macrophage cell line RAW264.7 [11]. Although the latter study implicated AGE-induced activation of NFκB in the upregulation of HO-1 expression, the effect of AGEs on the redox sensitive Nrf2-Keap1 pathway in endothelial cells has not been investigated. This study in bovine aortic endothelial cells demonstrates that AGE-modified bovine albumin, but not native albumin, evokes acute (30 min) and long-term (12—24 h) ROS generation, enhances Nrf2 nuclear translocation and JNK phosphorylation, leading to a time-dependent upregulation of HO-1 and NQO1 mRNA and protein expression.

Methods

Endothelial cell culture

Bovine aortic endothelial cells (BAEC) were isolated from fresh aortae using 0.5 mg ml^{-1} collagenase (Boehringer) and cultured in DMEM containing 10% fetal calf serum (FCS), 5.5 mM D-glucose, 5 mM L-glutamine, penicillin/streptomycin (100 IU ml^{-1}) and maintained at 37 °C under 5% CO_2 humidified atmosphere [12]. Endothelial phenotype was confirmed by a characteristic cobblestone morphology and positive immunostaining for von Willebrand factor and eNOS in cells in passage 3—8 (data not shown).

Preparation of AGE-modified bovine serum albumin

AGE-modified bovine serum albumin (AGE-BSA) was prepared by incubating bovine albumin BSA (10 mg ml^{-1}, fatty acid free) at 37 °C for 6 weeks with D-glucose (90 g L^{-1}) (Sigma, U.K.) in a 0.4-M phosphate buffer containing azide [13]. Native BSA preparations were treated identically except that glucose was omitted. Preparations were dialysed extensively against phosphate buffer to remove free glucose. When the extent of advanced glycation was assessed, AGE-BSA had a 3-fold lower content of free amines, 10-fold higher formation of carbonyl groups and 2-fold higher protein bound hydroperoxides compared with native BSA (Table 1). BSA and AGE-BSA concentrations (100 μg ml^{-1}) were based on previous studies [14,15], and cell viability was unaffected following acute or long-term treatments (data not shown).

Chemiluminescence detection of ROS in intact bovine aortic endothelial cells

ROS generation was measured in intact BAEC using the luminol analogue L-012 (8-amino-5-chloro-7-phenylpyridol[3,4-d] pyridazine-1,4-(2H,3H)dione sodium salt) [16,17]. We confirmed that neither BSA or AGE-BSA in buffer in a cell-free system affected L-012 chemiluminscence. Confluent cells were equilibrated in DMEM (5.5 mM D-glucose, 1% FCS) for 24 h and then treated for 0—24 h with DMEM (control) or DMEM containing BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of the superoxide chelator Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, 10 μM) [18], inhibitors of eNOS (L-NAME, 100 μM), flavoproteins (diphenylene iodonium, DPI, 10 μM) or NADPH oxidase subunit assembly (apocynin,

Table 1 Extent of glycation of bovine serum albumin.

Assay	Measurement	BSA	AGE-BSA
Free amines	Absorbance 335 nm	0.872 ± 0.026	0.299 ± 0.016
Carbonyls (moles carbonyls/mol albumin)	HPLC analysis of derivatised samples	0.102 ± 0.003	0.854 ± 0.019
Protein bound hydroperoxides (mmol ROOH/mol albumin)	Fox 1 assay	5.09 ± 0.95	9.49 ± 0.68

Data denote means ± S.E.M.

4′-hydroxy-3'methoxyacetophenone, 100 μM) [19], or mitochondrial complex I (rotenone, 5 μM) [20]. Inhibitor concentrations were based on our previous studies with vascular smooth muscle cells [17,21]. Cells were incubated at 37 °C in Krebs buffer containing BSA, AGE-BSA (in the continued absence or presence of BSA, AGE-BSA and/or inhibitors) and L-012, with luminescence monitored immediately over 40 min after addition of L-012. Mean light units were recorded in a microplate luminometer (Chameleon V, Hidex), and maximal values obtained over a 20—40 min interval averaged for each treatment condition in 3—4 independent cell cultures and expressed as mean light units/μg protein.

Quantitative RT-PCR

Cells were treated for specified times with DMEM (control) or DMEM containing BSA (100 μg ml^{-1}), AGE-BSA (100 μg ml^{-1}) or diethylmaleate (DEM, 100 μM), known to activate Nrf2-dependent gene expression in macrophages and vascular smooth muscle cells [7,22]. Total RNA was isolated using the Nucleospin 96-well plate format RNA isolation kit (Macherey—Nagel). Cells were lysed, RNA purified using Macherey—Nagel RNA extraction kit, quantified and reverse-transcribed using QuantiTect RT kit (Qiagen). Expression of HO-1, NQO1 and glutathione peroxidase-1 (GPx-1) was analyzed using a quantitative RT-PCR system (Corbett Rotor-gene), and mRNA levels were normalized to the geometric mean of four stable house-keeper genes, RPL13, β-actin (ACTB), β-microglobulin (β2M), and TATA box binding protein (TBP) [23] (Table 2).

Immunoblotting

Cells were treated for 6—24 h with DMEM (control) or DMEM containing BSA (100 μg ml^{-1}), AGE-BSA (100 μg ml^{-1}) or DEM (100 μM). Treatments were stopped by washing cells with ice-cold phosphate buffered saline and cells lysed in buffer containing protease and phosphatase inhibitor cocktails (Sigma). Nuclear extracts were prepared following cell lysis in 0.5% Nonidet P-40 containing buffer, as described previously [21,22]. Protein content was determined using the bicinchoninic acid assay (BCA, Pierce), and lysates were subjected to gel electrophoresis and immunoblotted with antibodies against HO-1 (Stressgen), NQO1, GPx-1, Nrf2, or phosphorylated isoforms of ERK1/2 (Promega), JNK and $p38^{MAPK}$ (Cell Signaling). α-tubulin (Chemicon International) and Lamin A/C (Santa Cruz) were used as loading controls. Enhanced chemiluminescence was used to visualize bands on autoradiographic film (Amersham, UK) and quantified using Image J software (National Institute of Health, USA)

Nrf2 localization by immunofluorescence

BAEC grown on Lab-Tek II chamber slides were treated with DMEM (control) and DMEM containing BSA (100 μg ml^{-1}), AGE-BSA (100 μg ml^{-1}) or DEM (100 μM). Cells were washed, fixed

Table 2 Bovine primer sequences for qRT-PCR measurements.

Gene Name	Gene Bank Accession No.	Primer	Sequence (5′ to 3′)
House-keeper genes			
TBP	NM_001075742	Forward	TTGTGCTTACCCACCAACAG
		Reverse	GGAGAACAATTCTCGGTTTGA
ACTB	NM_173979.3	Forward	CAAGGCCAACCGTGAGAA
		Reverse	GTACATGGCAGGGGTGTTG
RPL13	NM_001076998.1	Forward	TCCCACCACCCTATGACAAG
		Reverse	GAGTAGGCTTCAGACGCACA
β2M	NM_173893	Forward	CCACCCCAGATTGAAATTGA
		Reverse	CAGGTCTGACTGCTCCGATT
Antioxidant genes			
HO-1	NM_001014912	Forward	AGACTTGGCTCCCACCAA
		Reverse	AGGTGCCTGGGAGAGGAC
GPx-1	NM_174076	Forward	CAACCAGTTTGGGCATCAG
		Reverse	GGACGTACTTCAGGCAATTCA
NQO1	NM_001034535	Forward	CGGAATAAGAAGGCAGTGCT
		Reverse	CCATGGATACCATGCAGAGA

in PBS containing paraformaldehyde (4%), permeabilized with Triton X-100 (0.1%) and examined by immunofluorescence using an anti-Nrf2 primary antibody (Santa Cruz) and Alexa Fluor 488-labeled secondary antibody (Invitrogen). Nuclei were co-labeled with propidium iodide (500 ng ml^{-1}, 15 min) prior to visualizing cells. An inverted microscope (Nikon, Eclipse TE2000-U) fitted with appropriate fluorescence filters and a CCD digital camera (Nikon, DXM1200F) was used to capture images [21].

Cell culture reagents and inhibitors

All cell culture reagents, diphenylene iodonium, apocynin, Tiron, rotenone, diethylmaleate and L-NAME were obtained from Sigma (UK); L-012 from Wako Pure Chemical Industries Ltd (Japan); SB203580 from Alexis (UK) and SP600125 from Biomol (UK).

Statistical analysis

Data are expressed as means ± S.E.M. with variance from the mean determined using the normal distribution with confidence limits established using a paired Student's *t*-test and two-way ANOVA for comparison of multiple groups. $P < 0.05$ was considered statistically significant.

Results

AGE-BSA and electrophilic stress upregulate HO-1 and NQO1 expression in BAEC

Treatment of BAEC for 4–12 h with AGE-BSA (100 μg ml^{-1}), but not BSA (100 μg ml^{-1}), results in a significant increase in mRNA levels for HO-1 (Fig. 1A) and NQO1 (Fig. 1B), whereas expression of GPx-1 was unaffected (data not shown). As induction of HO-1 by DEM is markedly attenuated in Nrf2-deficient vascular cells [22], we examined whether DEM mimicks the effects of AGE-BSA on gene expression. As shown in Fig. 1C, treatment of cells for 8 h with DEM (100 μM) increased mRNA levels for HO-1 (10-fold) and NQO1 (6-fold) but had no effect on expression of GPx-1, a redox sensitive gene regulated independently of Nrf2 [24].

Treatment of BAEC with AGE-BSA (100 μg ml^{-1}), but not BSA, induced in a time-dependent increase in HO-1 protein levels, which were maximal after 24 h treatment (Fig. 1D). GPx-1 protein levels were unaffected after treatment of cells for 6–24 h with BSA or AGE-BSA (data not shown), consistent with the lack of GPx-1 mRNA induction in response to AGE-BSA or DEM (Fig. 1C). The Nrf2 inducer DEM (100 μM) evoked a time-dependent increase in HO-1 protein

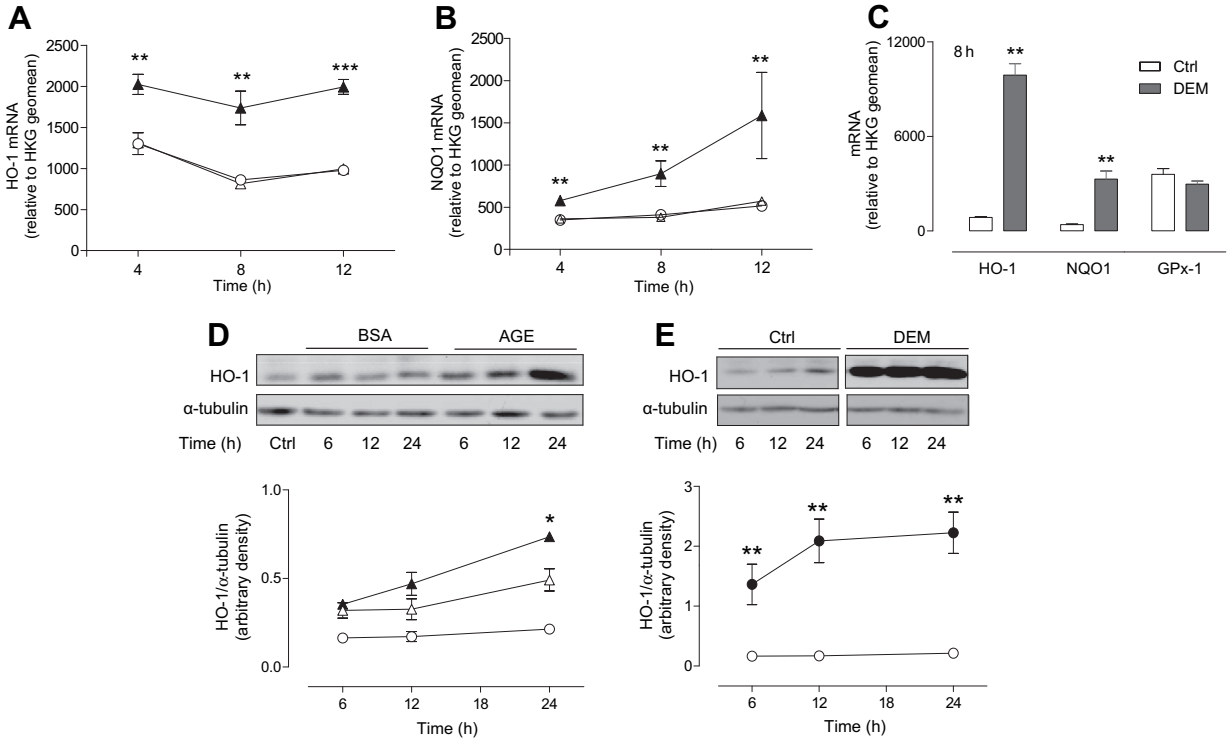

Figure 1 AGE-BSA upregulates expression of Nrf2/ARE-linked genes HO-1 and NQO1 in BAEC. Confluent BAEC monolayers were equilibrated with DMEM (5.5 mM D-glucose, 1% FCS) and then treated for 4, 8, and 12 h with DMEM (control, ○-○) or DMEM containing BSA (100 μg ml^{-1}, △-△) or AGE-BSA (100 μg ml^{-1}, ▲-▲). A-B, Time course of HO-1 and NQO1 mRNA expression. C, HO-1, NQO1 and GPx-1 mRNA expression after 8 h treatment with diethylmaleate (DEM, 100 μM), a well known inducer of Nrf2 mediated gene transcription. Target gene expression was normalized to the geometric mean of four stable house-keeper genes (HKG Geomean, see Table 2). D-E, immunoblots and densitometric analysis of HO-1 expression (relative to α-tubulin) in BAEC treated with DMEM (○-○), BSA (△-△), AGE-BSA (▲-▲) or DEM (●-●). Data denote means ± S.E.M., $n = 4$ independent experiments in different BAEC cultures, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, AGE-BSA vs. control (Ctrl) and BSA; $^{**}P < 0.01$ DEM vs. control (Ctrl).

levels, with maximum expression detected after 12–24 h treatment (Fig. 1E).

Nrf2 nuclear translocation endothelial cells treated with AGE-BSA and diethylmaleate

As nuclear translocation of Nrf2 is associated with transcriptional activation of the ARE-linked genes HO-1 and NQO1 [7,22], cells were treated for 1, 2 and 4 h with BSA, AGE-BSA or DEM and nuclear accumulation of Nrf2 determined by immunoblotting and immunofluorescence. AGE-BSA (100 μg ml^{-1}) and DEM (100 μM) enhanced nuclear accumulation of Nrf2 (Fig. 2A) and immunofluorescence analysis confirmed that Nrf2 was predominantly localized in the cytosol in cells incubated in DMEM, whereas AGE-BSA (100 μg ml^{-1}) or DEM (100 μM) enhanced nuclear translocation of Nrf2 and co-localization with propidium iodide (Fig. 2B).

AGE-BSA stimulates acute and long-term ROS production in intact BAEC monolayers

Although previous studies have shown that AGEs enhance ROS production in endothelial cells [3,25], a time course of AGEs induced ROS generation has not been reported. When ROS production was monitored after treating BAEC for 30 min, 12 h or 24 h with BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}), L-012 luminescence increased significantly after 30 min and elevated ROS generation was maintained over 12–24 h treatment (Fig. 3A). In time control experiments, DMEM and BSA had negligible effects on ROS production.

To further examine sources of intracellular ROS generation, cells were treated for 24 h with BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of inhibitors of flavoproteins (diphenylene iodonium) and NADPH oxidase subunit assembly (apocynin) or a superoxide scavenger (Tiron). AGE-BSA stimulated ROS production over 24 h was abrogated by DPI (10 μM), apocynin (100 μM) and Tiron (10 μM) (Fig. 3B), implicating NADPH oxidase in ROS production. In contrast, inhibition of mitochondrial complex I with 5 μM rotenone (Fig. 3C) or eNOS with 100 μM L-NAME (Fig. 3D) had negligible effects on AGEs mediated ROS generation.

Activation of NADPH oxidase contributes to AGE-BSA induced HO-1 expression

To determine whether inhibition of ROS generation via NADPH oxidase affects AGEs induced HO-1 expression, cells

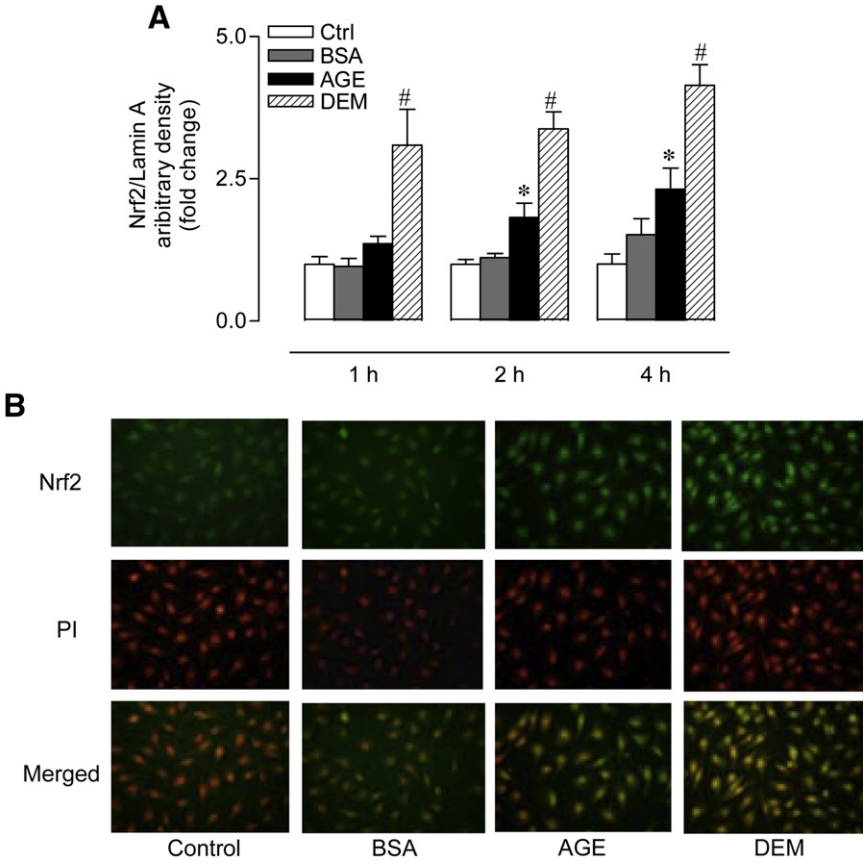

Figure 2 AGE-BSA induces Nrf2 nuclear translocation in BAEC. Cells equilibrated for 24 h in DMEM were treated for 1, 2, and 4 h with DMEM (Ctrl) or DMEM containing BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) or DEM (100 μM). A, densitometric analysis of nuclear Nrf2 protein levels relative to Lamin A/C (loading control). Means ± S.E.M., $n = 3$, $^{*}P < 0.05$, AGE-BSA vs. control (Ctrl, 5 mM D-glucose) or native BSA; $^{\#}P < 0.01$, DEM vs. control (Ctrl). B, Cells were treated for 2 h with DMEM (Ctrl), BSA, AGE-BSA or DEM and Nrf2 localized using an anti-Nrf2 antibody and Alexfluor-conjugated secondary antibody, with cell nuclei co-stained with propidium iodide (PI). All panels × 10 magnification.

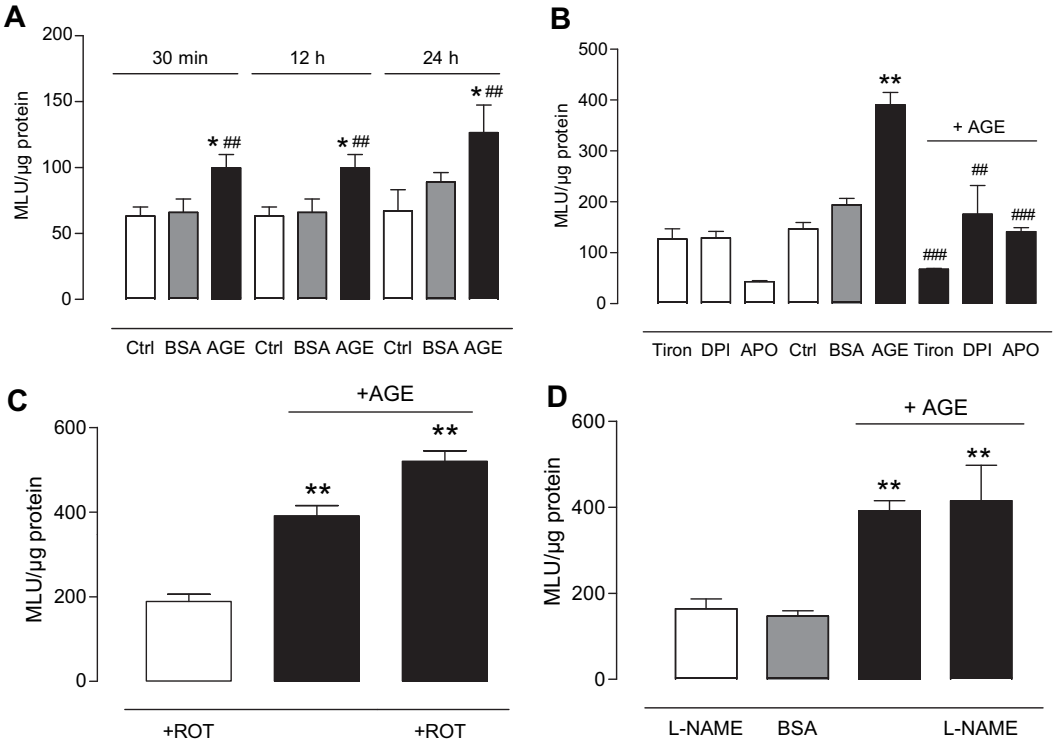

Figure 3 AGE-BSA stimulates acute and long-term ROS production in intact BAEC. A, Cells were treated for 30 min, 12 h or 24 h with DMEM (Ctrl), BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}). ROS generation was measured immediately over 0—40 min after incubating cells in Krebs buffer containing L-012 (100 μM) in the continued absence or presence of BSA, AGE-BSA. Means ± S.E.M. of triplicate measurements in 4 independent cultures, $^{*}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Ctrl or BSA, respectively. B, AGE-BSA (100 μg ml^{-1}) stimulated ROS production after 24 h co-treatment with Tiron (10 μM), DPI (10 μM) or apocynin (APO, 100 μM). Means ± S.E.M., $n = 4$, $^{**}P < 0.01$ AGE-BSA *vs* BSA or Ctrl; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ AGE-BSA + inhibitors *vs* AGE-BSA alone. C, ROS generation in cells after 24 h treatment with BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of rotenone (ROT, 5 μM, vehicle 0.1% DMSO). Means ± S.E.M., $n = 4$, $^{**}P < 0.01$ AGE-BSA *vs* rotenone alone. D, ROS generation in cells after 24 h treatment with BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of L-NAME. Means ± S.E.M., $n = 3$, $^{**}P < 0.01$ *vs* BSA alone.

were treated for 24 h with BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of DPI (10 μM), apocynin (100 μM) or Tiron (10 μM). Upregulation of HO-1 expression by AGE-BSA was inhibited by DPI, apocynin and Tiron (Fig. 4A) but affected negligibly by 5 μM rotenone (Fig. 4B) or 100 μM L-NAME (Fig. 4C).

Activation of intracellular kinase pathways in BAEC by AGE-BSA

Our previous studies in vascular smooth muscle cells implicated kinase signaling pathways in Nrf2 mediated upregulation of HO-1 [21], and AGEs have been shown to activate p38, extracellular signal-regulated kinase 1/2 and Jun N-terminal kinase in different cell types [11]. To elucidate whether one or more of these kinase pathways mediates AGEs induced HO-1 expression in BAEC, we examined acute (5—30 min) phosphorylation of p38^{MAPK}, ERK1/2 and JNK. Only JNK was activated by AGEs (Fig. 5A,B), while phosphorylation of p38^{MAPK} and ERK1/2 was unaffected (data not shown). Although AGEs stimulated Akt phosphorylation (Fig. 5C,D), inhibition of upstream PI3-kinase with LY294,002 (10 μM) had negligible effects on AGEs induced HO-1 expression (data not shown), whereas inhibition of JNK with SP600125 (20 μM) abrogated stimulated HO-1 protein levels (Fig. 5E,F).

Discussion

AGEs and their intermediates contribute to vascular complications associated with diabetes, increased oxidative stress and activation of many transcription factors and their downstream target genes. Previous studies focused primarily on AGEs induced pro-inflammatory processes involving NFκB [10,26] and have not characterised the potential involvement of other transcription factors such as Nrf2 in the induction of the antioxidant gene HO-1. This is the first study documenting that AGEs upregulate Nrf2/ARE-linked antioxidant and phase II defense gene expression in endothelial cells.

Increased oxidative stress induced by the interaction of AGEs with RAGE [4] has been documented in many cell types including endothelial cells, with activation of NADPH oxidase previously implicated in AGEs induced oxidative stress and altered NFκB-dependent gene expression [4,10,11,25]. We found that AGEs increased ROS generation within 30 min and report that increased superoxide production is sustained over 12—24 h

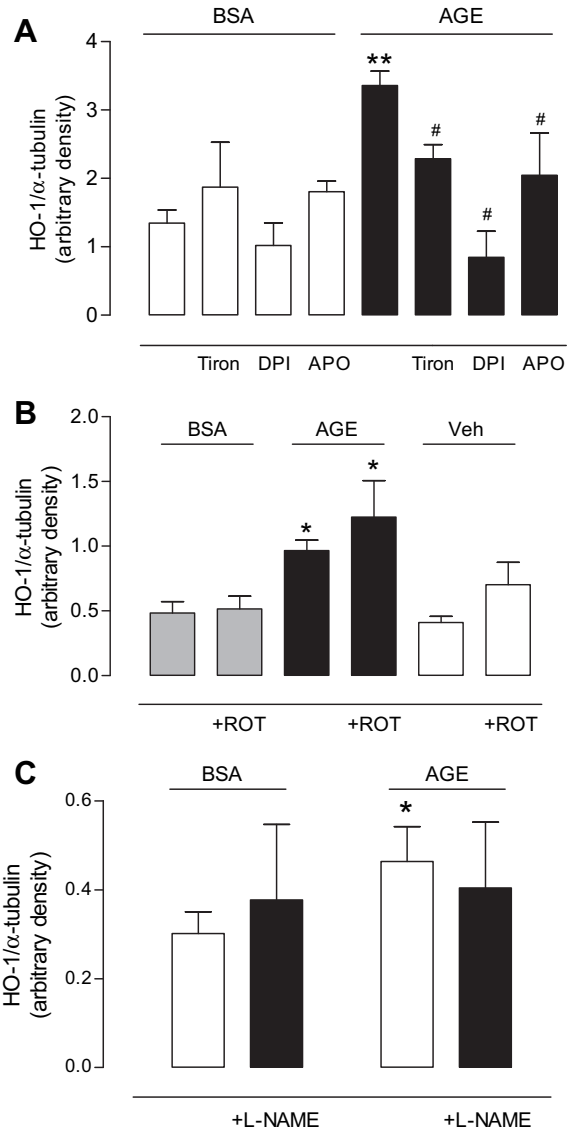

Figure 4 Effect of inhibitors of ROS generating pathways on AGE-BSA induced HO-1 expression. BAEC were treated for 24 h with native BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of (A) DPI (10 μM), apocynin (100 μM), Tiron (10 μM), (B) rotenone (5 μM) or (C) L-NAME (100 μM). Immunoblots for HO-1 expression were analyzed, means ± S.E.M., $n = 3–4$, $^{*}P < 0.05$, $^{**}P < 0.01$ AGE-BSA *vs* BSA alone; $^{\#}P < 0.05$, AGE-BSA + inhibitors *vs* AGE-BSA alone.

treatment and associated with Nrf2/ARE mediated gene transcription. Our findings are consistent with a recent study in human endothelial cells where transient hyperglycemia induced a long-lasting activation of NFκB-linked proatherogenic gene expression involving increased ROS generation [27]. 'Pre-conditioning' stimuli that activate redox signaling pathways may also provide cytoprotection against sustained oxidative damage via activation of Nrf2. In our study, superoxide scavengers and inhibitors of flavoproteins and/or NADPH oxidase abrogated acute (30 min) and longer-term (12–24 h) ROS production (2–2.8-fold) in response to AGEs, confirming previous studies assaying acute ROS production in human umbilical vein and bovine retinal endothelial cells [25,28]. As inhibition of eNOS or the mitochondrial respiratory chain had no significant effect on AGEs induced ROS production or HO-1 expression, it seems unlikely that ROS released via these pathways mediate Nrf2/ARE-linked gene expression in BAEC.

Upregulation of HO-1 and NQO1 mRNA and protein expression in BAEC challenged with AGEs was associated with Nrf2 nuclear translocation. AGEs had negligible effects on expression of GPx-1 (data not shown), an antioxidant enzyme regulated independently of Nrf2. As DEM also increased nuclear translocation of Nrf2 and HO-1 and NQO1 expression, but not GPx-1, this further implicates the Nrf2-Keap1 pathway in adaptive responses of BAEC to AGEs induced oxidative stress.

Regulation of HO-1 gene activation occurs at multiple levels and is inducer specific [29]. The cytoprotective properties of HO-1 may be due to bilirubin directly inhibiting NADPH oxidase activity by interrupting subunit assembly [6], potentially implicating AGEs induced HO-1 expression in the downregulation of NADPH oxidase activity. Carbon monoxide (CO) shares some properties of nitric oxide (NO) in modulating intracellular signaling processes, including dilatory, anti-inflammatory, antiproliferative, antiapoptotic, and anticoagulative effects [29,30]. NQO1 is a cytoplasmic two electron reductase that catalyzes reduction of a wide range of substrates including quinones, quinone-imines, and nitro-compounds [31]. Thus, upregulation of HO-1 and NQO1 in response to AGEs and other stimuli provides an effective endogenous antioxidant defense mechanism in diabetes and other vascular diseases [6].

Studies in other cell types have shown that induction HO-1 via Nrf2 is in part mediated by the activation of intracellular protein kinase cascades, with the MAPKs playing a major role. We previously reported that induction of HO-1 expression in human aortic smooth muscle cells by oxidized LDL or TGF-β1 is associated with an increase in phosphorylation of p38^{MAPK}, ERK and JNK [17,21], and here report that inhibition of kinase signaling via JNK abrogates AGE-BSA induced HO-1 expression. NADPH oxidase derived superoxide and activation of JNK may underlie AGEs induced activation of HO-1 via Nrf2. The p47^{phox}-dependent NADPH oxidase isoform Nox2 has been shown to be involved in JNK activation [32]. Moreover, Nox2 requires phosphorylation of the p47^{phox} subunit by kinases such as Akt [33], which we found was activated in BAEC treated with AGEs. However, as inhibition of PI3-kinase/Akt signaling with LY294,002 did not significantly inhibit AGEs stimulated gene expression (data not shown), it seems unlikely that PI3-kinase mediates AGEs induced activation of the Nrf2-Keap1 pathway. Our findings are consistent with a report that inhibition of PI3-kinase with wortmannin and LY294002 fails to inhibit HO-1 induction in response to unsaturated aldehydes [34].

Several transcription factors, including c-Jun, ATF2, and ATF4, are implicated as potential partners of Nrf2 in electrophile (EpRE)-mediated induction of antioxidant genes and, as recently reported, the phosphorylated form c-Jun may predominate in a given cell type and the effects of phosphorylation of c-Jun on gene transcription may vary [35]. This variability in the response among different genes

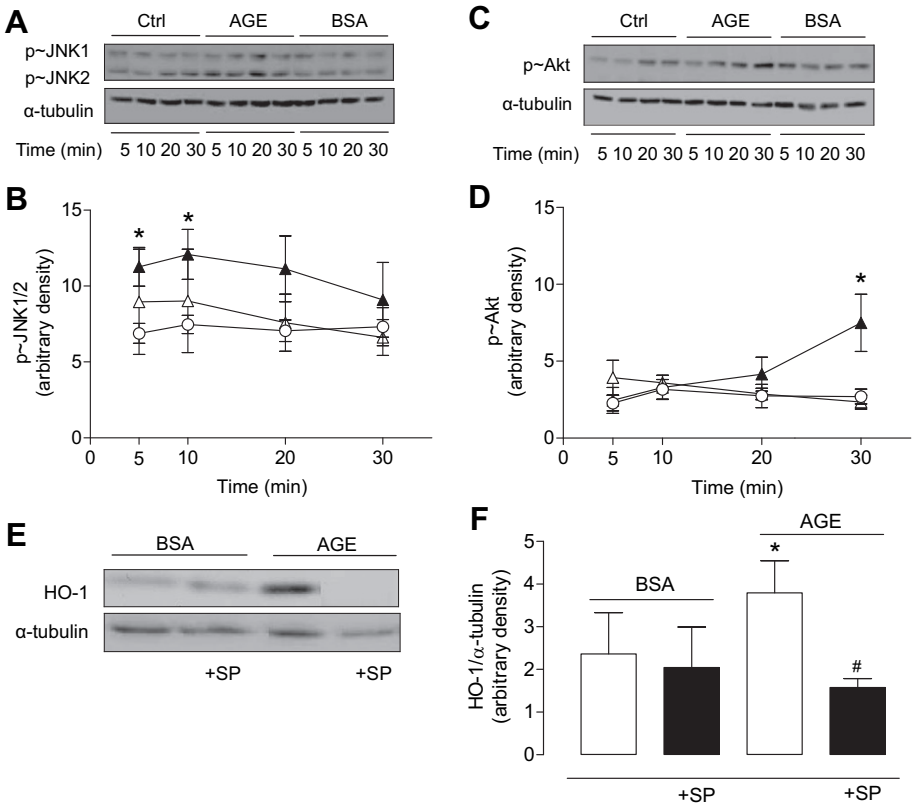

Figure 5 Activation of the JNK signaling pathway mediates AGE-BSA induced HO-1 expression in BAEC. Cells were treated for 5, 10, 20, and 30 min with DMEM (control, ○-○) or DMEM containing BSA (100 μg ml^{-1}, △-△) or AGE-BSA (100 μg ml^{-1}, ▲-▲). A-B, Representative immunoblots of the time course of AGE-BSA stimulated JNK1/2 and Akt phosphorylation. C-D, Densitometric analysis of p ~ JNK1/2 and p ~ Akt by AGE-BSA relative to control or BSA. E, HO-1 protein levels in cells treated for 24 h with BSA (100 μg ml^{-1}) or AGE-BSA (100 μg ml^{-1}) in the absence or presence of the JNK inhibitor SP600125 (SP, 20 μM). F, Densitometric analysis of SP600125 mediated inhibition of AGE-BSA induced HO-1 expression. Means ± S.E.M., $n = 4$, $^{*}P < 0.05$, AGE-BSA *vs* BSA or control, $^{\#}P < 0.05$, AGE-BSA *vs* AGE plus SP600125.

suggests that the same cis element may be regulated differentially not only in a given gene but also in one cell type versus another [35]. This may, in part, account for the differential time course for AGEs induced HO-1 and NQO1 mRNA and protein expression in BAEC observed in the present study.

Activation of NADPH oxidase by AGEs may act as a double-edged sword, with low ROS levels activating the redox sensitive Nrf2-Keap1 pathway to restore redox homeostasis, and an overproduction of ROS leading to uncoupling of eNOS, mitochondrial dysfunction and impaired redox signaling [6]. Feedback inhibition of NADPH oxidase by HO-1 derived bilirubin could provide a mechanism by which activation of the Nrf2-Keap1 pathway by AGEs leads to vascular protection to counteract oxidative stress in diabetes.

Conflict on interest

The authors do not have conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) this work.

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