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Amadori adducts activate nuclear factor-κB-related proinflammatory genes in cultured human peritoneal mesothelial cells

*,[1]Julián Nevado, [2]Concepción Peiró, [1]Susana Vallejo, [1]Mariam El-Assar, [2]Nuria Lafuente, [2]Nuria Matesanz, [2]Verónica Azcutia, [2]Elena Cercas, [2]Carlos F. Sánchez-Ferrer & [1,3]Leocadio Rodríguez-Mañas

[1]Unidad de Investigación, Hospital Universitario de Getafe, Ctra. de Toledo Km 12.5, Getafe, Madrid 28905, Spain; [2]Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid 28029, Spain and [3]Servicio de Geriatría, Hospital Universitario de Getafe, Madrid 28905, Spain

1 Diabetes mellitus leads to a high incidence of several so-called complications, sharing similar pathophysiological features in several territories. Previous reports points at early nonenzymatic glycosylation products (Amadori adducts) as mediators of diabetic vascular complications. In the present study, we analysed a possible role for Amadori adducts as stimulators of proinflammatory pathways in human peritoneal mesothelial cells (HPMCs).

2 Cultured HPMCs isolated from 13 different patients (mean age 38.7±16 years) were exposed to different Amadori adducts, that is, highly glycated haemoglobin (10 nM) and glycated bovine serum albumin (0.25 mg ml^{-1}), as well as to their respective low glycosylation controls. Amadori adducts, but not their respective controls, elicited a marked increase of NF-κB activation, as determined by electromobility shift assays and transient transfection experiments.

3 Additionally, Amadori adducts significantly increased the production of NF-κB-related proinflammatory molecules, including cytokines, such as TNF-α, IL-1β or IL-6, and enzymes, such as cyclooxygenase-2 and inducible nitric oxide (NO) synthase, this latter leading to the release of NO by HPMCs.

4 The effects of Amadori adducts were mediated by different reactive oxygen and nitrosative species (e.g. superoxide anions, hydroxyl radicals, and peroxynitrite), as they were blunted by coincubation with the appropriate scavengers. Furthermore, NO generated upon exposure to Amadori adducts further stimulated NF-κB activation, either directly or after combination with superoxide anions to form peroxynitrite.

5 We conclude that Amadori adducts can favour peritoneal inflammation by exacerbating changes in NO synthesis pathway and triggering NF-κB-related proinflammatory signals in human mesothelial cells.
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Abbreviations: AP-1, activator protein-1; AGEs, advanced glycation end products; CAPD, continuous ambulatory peritoneal dialysis; DMTU, dimethylthiourea; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; eNOS, constitutive endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HHb, highly glycated oxyhaemoglobin; HPMCs, human peritoneal mesothelial cells; iNOS, inducible nitric oxide synthase; L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; NF-κB, nuclear factor-κB; NHb, oxyhaemoglobin glycated at normal levels; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase; Tempol, 4-hydroxy-tempo

Introduction

Diabetic patients account for approximately 40% of patients undergoing dialysis in Western Europe and United States (Stein *et al*., 2004). At the same time, the use of continuous ambulatory peritoneal dialysis (CAPD) is increasing as a renal replacement therapy in these patients. Although the influence of diabetes on peritoneal membrane (PM) remains debated, long-term hyperglycaemia has been suggested to be involved in several changes affecting the functionality of the PM, either in animal models or patients undergoing CAPD (Nakamoto *et al*., 2002; Stoenoiu *et al*., 2002). Indeed, such alterations of the peritoneum could be responsible, at least in part, for the higher mortality rate observed in diabetic patients compared to nondiabetic patients with peritoneal dialysis (Stein *et al*., 2004).

In recent years, alterations of peritoneal mesothelial cells have been proposed to be on the basis of PM dysfunction in these patients (Yañez-Mo *et al*., 2003; Yao *et al*., 2003). PM includes a monolayer of mesothelial cells, which acts as a permeability barrier. These cells share many properties with

*Author for correspondence; E-mail: jnevado@hug.es

vascular endothelial cells, including the ability to synthesise nitric oxide (NO) and prostacyclin (Amore *et al*., 1997). Recent studies suggest that some pathophysiological mechanisms involved in diabetic microvascular complications may also be participating in PM dysfunction. Among them, an increased local release of growth factors (Mandl-Weber *et al*., 2002), and proinflammatory mediators (Riese *et al*., 1999), as well as deregulation of NO synthases (Devuyst *et al*., 2001) and increased accumulation of advanced glycosylation end products (AGEs) (Park *et al*., 2000) within the peritoneum is worth noting.

In the last years, several groups, including ours, have postulated that other products of nonenzymatic protein glycosylation, different to AGEs, can play an important role in diabetic vascular complications. Amadori adducts are the result of condensation reactions between glucose and reactive protein amino groups, which yield Schiff bases that undergo reversible rearrangement within days or weeks. Amadori adducts can, in turn, undergo irreversible changes to form AGEs after longer periods of time (Cerami *et al*., 1988). In the vasculature, circulating Amadori adducts, like glycated haemoglobin or glycated albumin, produce reactive oxygen species (ROS), which in turn inactivate NO, leading to endothelial dysfunction (Angulo *et al*., 1996; Amore *et al*., 1997; Peiró *et al*., 1998; Hattori *et al*., 1999; Vallejo *et al*., 2000a; Peiró *et al*., 2001; Rodríguez-Mañas *et al*., 2003). Amadori adducts can also activate proinflammatory redox-regulated transcription factors, including NF-κB (Hattori *et al*., 1999; 2002; Mandl-Weber *et al*., 2001; Peiró *et al*., 2003).

Alterations of the PM are associated with a local proinflammatory environment that may be triggered by different molecules, including the above-mentioned ROS, NO, or NF-κB. In the present work, we aimed to study the ability of Amadori adducts to induce a proinflammatory response in human peritoneal mesothelial cells (HPMCs). For this purpose, we tested the effects of both glycated haemoglobin and glycated albumin on NF-κB activity, *inducible NO synthase (iNOS)* and *cyclooxygenase-2 (COX-2)* gene expression and activity, as well as on the secretion and gene expression of several proinflammatory cytokines such as IL-1β, IL-6, and TNF-α by HPMCs. In addition, a putative role for ROS and reactive nitrosative species was also analysed.

Methods

Materials

Culture plastic ware was obtained from Corning-Costar (New York, NY, U.S.A.). M199 medium, L-glutamine, and streptomycin/penicillin solutions were purchased from Biochrom KG, Berlin, Germany. Phosphate-buffered saline (PBS), foetal calf serum (FCS) and trypsin–EDTA were from Amresco (Solon, Ohio, U.S.A.), Biological Industries (Beit-Hamek, Israel), and GIBCO BRM (Paisley, U.K.), respectively. Human TNF-α was from Peprotech (London, U.K.) and IL-1β from R&D systems (Minneapolis, MN, U.S.A.). *Taq* DNA polymerase and dNTPs were from EGOGEN (Barcelona, Spain), while 1400W ([*N*-(3-aminomethyl) benzylacetamidine, 2 HCl]) was from Calbiochem (Darmstadt, Germany). Unless otherwise stated, all other reagents were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Preparation of Amadori adducts

Lyophilised human haemoglobins, nonenzymatically glycosylated at either elevated or normal levels, containing 11.1% (Catalogue no. G-1012) and 5.4% (Catalogue no. G-2012) HbA_1, respectively, were purchased from Sigma Chemical Co. Before use, haemoglobins were prepared as described previously (Peiró *et al*., 2003). Briefly, haemoglobins were dissolved in deionised water and subsequently reduced by incubation with an excess of sodium dithionite. The haemoglobin solutions were then extensively dialysed using a 0.25 Å pore diameter (approximately 12 kDa mol wt) dialysis membrane (Visking®, Serva, Heidelberg, Germany) against deionised water containing $10\,mg\,l^{-1}$ EDTA and continuously bubbled with N_2. Oxyhaemoglobins were then aliquoted and stored at −70°C until used.

A $50\,mg\,ml^{-1}$ solution of bovine serum albumin (BSA) (Sigma Chemical Co.) was glycosylated by incubation in PBS (pH 7.4) containing 1 M glucose under sterile and light-protected conditions for 6 days at 37°C as described previously (Peiró *et al*., 1998). A control solution was prepared in parallel using PBS without glucose. Glycosylation of serum albumin was verified using the thiobarbituric acid assay (Ney *et al*., 1981).

The absence of AGEs in the glycated oxyhaemoglobin and albumin solutions was assessed by measuring fluorescence in a Fluostar fluorometer (BMG Labtechnologies, Offenburg, Germany) at excitation maximum of 370 nm and emission maximum of 440 nm, which allows quantifying total AGEs (Sell & Monnier, 1989). A standard curve ($r = 0.99$) was carried out using AGE-modified BSA (0.5–5 $\mu g\,ml^{-1}$), prepared following a previously described method (Bucala *et al*., 1991). Glycated preparations did not contain significative bacterial endotoxin contamination ($\leqslant 0.5$ U endotoxin ml^{-1}), as measured with Pyrogent® plus kit (Biowhittaker Europe SPRL, Verners, Belgium).

Cell culture

HPMCs were isolated from omental tissue from 13/15 different donors (free of any cardiovascular or peritoneal disease and nontaking anti-inflammatory drugs or antioxidants) undergoing nonurgent, nonseptic abdominal surgery, using previously described methods (Chung-Welch *et al*., 1997). HPMCs were routinely cultured in M199 containing $1\,g\,l^{-1}$ of D-glucose and supplemented with 10% FCS, $100\,\mu g\,ml^{-1}$ streptomycin, $100\,U\,ml^{-1}$ penicillin, and $2.5\,\mu g\,ml^{-1}$ amphotericin. At confluence, HPMC were passaged using a 0.02% EDTA–0.05% trypsin solution and split in a 1 : 2 ratio. HPMCs characterisation was based on both cell morphology (immediately prior to and at confluence, cells adopted the polygonal cobblestone-like appearance characteristic of epithelial cells and formed a monolayer) and indirect immunofluorescence staining of several human mesothelial markers (Chung-Welch *et al*., 1997). In brief, HPMCs showed a diffuse positive staining with an anti-von Willebrand factor antibody (Dakopatts, Glostrup, Denmark) and a marked staining with anti-cytokeratins 8 and 18, anti-E-cadherin, and anti-vimentin antibodies (all of them from Sigma Chemical Co.). HPMCs failed to express the endothelial marker PECAM-1 (CD31) (see Supplemental data, Figure 1). Cell cultures between passages two and eight were used.

Reporter plasmids

The reporter plasmid, p5 × NF-κB-Luc (Stratagene, La Jolla, CA, U.S.A.), and different luciferase-based reporter plasmids corresponding to the 5′-flanking regulatory regions of either human *iNOS* (7.2 hiNOS-luc; Taylor *et al*., 1998), human *eNOS* (1.33 heNOS-luc; Cieslik *et al*., 1999), short human *COX-2* and human *COX-1* (phPES2 −327/+59, phPES1 −1010/+69, respectively; Inoue *et al*., 1995), or human *IL-6* (p1168hu.IL6P-luc; Berghe *et al*., 1998) genes were used.

Transient transfection and luciferase assays

Transient transfection experiments were performed as we described previously (Peiró *et al*., 2003). Briefly, HPMCs (10^5 cells) were grown in six-well plates to 80–90% confluence and the culture medium (M-199) was then replaced by vehicle medium, that is, serum-free medium supplemented with 0.1% BSA. The transfection mixture was added to cell cultures for further 18–20 h. The transfection mixture consisted of 2 μg of the above-mentioned plasmids incubated with 75 μl of DMEM and 7.5 μl of Superfect® (Quiagen Gmbh, Hilden, Germany) in vehicle medium, following the manufacturer's instructions. Following treatment with the specified agents, HPMCs were harvested and lysed with passive lysis buffer (1 ×; Promega, Madison, WI, U.S.A.), followed by one freeze/thaw cycle. The extracts were centrifuged for 30 s at 13,000 r.p.m. at 4°C, and assayed with a luciferase reporter system (Promega, Madison, WI, U.S.A.). Luciferase activity was expressed as relative luciferase units (RLUs; Peiró *et al*., 2003).

Western blotting and protein content

Extraction of protein homogenates and Western blotting were performed as described previously (Peiró *et al*., 2003). Briefly, HPMCs were extracted in lysis buffer containing 10 $\mathrm{mmol\,l^{-1}}$ Tris, pH 7.4, 1% SDS, 10 $\mathrm{mmol\,l^{-1}}$ orthovanadate, 2 $\mathrm{mmol\,l^{-1}}$ PMSF, and 12.5 $\mu\mathrm{g\,ml^{-1}}$ of aprotinin. Total protein extracts were diluted 3 : 1 in 4 × Laemmli's buffer and boiled for 5 min at 100°C. Proteins were equally loaded (10 $\mu\mathrm{g\,lane^{-1}}$), separated on 12% SDS–PAGE gels, and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Madrid, Spain). After blocking overnight at 4°C in 0.2% Tween-20 and 5% nonfat dry milk, the membrane was incubated for 1 h at room temperature with a monoclonal antibody against either COX-2 or iNOS (dilution 1/1000; BD Biosciences, Bedford, MA, U.S.A.), or an affinity-purified anti-nitrotyrosine polyclonal antibody (dilution 1/750; Alexis, Carlsbad, CA, U.S.A.) followed by incubation for 45 min with the respective anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (dilution 1/10,000; Chemicon, Temecula, CA, U.S.A.). Immunoreactive bands were detected using an enhanced chemiluminescence detection kit (Amersham, Arlington Hills, IL, U.S.A.) and quantified using Chemi-Imager 5.5 software from AlphaInnotec (San Leandro, CA, U.S.A.).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described by others (Schreiber *et al*., 1989). EMSAs were performed as described previously (Peiró *et al*., 2003). Nuclear extracts (5 μg of protein) were incubated in the presence of 3 μg of poly-dIdC together with a commercial double-stranded ^{32}P-labelled oligonucleotide encoding for the NF-κB consensus sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. DNA–protein complexes were electrophoretically separated and subjected to autoradiography. Bands were quantified by densitometry using NIH Image software.

Determination of cytokine levels

Cytokine levels in confluent cell (10^5 cells) culture supernatants were determined using human TNF-α, IL-1β, and IL-6 Instant ELISAs (The Bender Medsystems, Vienna, Austria), by generating a standard curve provided by the manufacturer and normalised to protein content (1 μg). Protein content of whole-cell extract and cell culture supernatants were determined using the BCA assay (Pierce, Rockford, IL, U.S.A.).

Measurement of nitrite plus nitrate

Confluent HPMCs (10^5 cells) were grown in six-well plates and nitrite plus nitrate production (NO_x), used as an indirect quantification of NO, was measured in cell supernatants by an ozone-chemiluminescence method (Fries *et al*., 2003), using an NO detector (NOA® 280 analyzer, Sievers, Boulder, CO, U.S.A.). A standard curve was generated by injections of known concentrations of sodium nitrate. The levels of NO_x were normalised to protein content (1 μg).

Measurement of COX activity

Confluent HPMCs (10^5 cells) were grown in six-well plates and COX activity was measured by the Cyclooxygenase Activity Kit (Stressgen Biotech, Madison, WI, U.S.A.) using a specific chemiluminescence substrate to detect the peroxidative activity of COX enzymes in protein extract homogenates, as specified by the manufactures. Light emission is directly proportional to COX activity in the sample. Results are expressed as RLUs normalised to protein content (1 μg).

RNA isolation and RT–Multiplex PCR (MPCR) assays

Total RNA from HPMC (10^5–10^6 cells) was obtained using RNAquous® kit (Ambion Inc., Austin, TX, U.S.A.), following the manufacturer's instructions. RT and MPCR were performed with appropriate kits (Maxim Biotech. Inc., San Francisco, CA, U.S.A.), using 1 μg of cDNA for each MPCR reaction. MPCR kit has been designed to direct the simultaneous amplification of specific ORF regions of human *NOS* genes and *GAPDH* (hNOSG-MPCR), human *COX* NF-κB (*NF-κB1, 2*) and *GAPDH* genes (hTNF-M052G-MPCR), or the proinflammatory cytokines *IL-6*, *IL-1β*, *TNF-α*, their respective receptors, and *GAPDH* genes (h-Inflammation-M053G-MPCR). Levels of mRNA were normalised to GAPDH transcript.

Ethic considerations

The study was approved by the Clinical Research and Ethics Committee of Hospital Universitario de Getafe, with oral informed consent obtained from all donors.

Statistical analysis

Results are expressed as mean ± s.e.m. Statistical analysis was determined by ANOVA, followed by Fisher's protected least-significance test with the level of significance chosen at $P \leqslant 0.05$. n denotes the number of experiments performed in triplicate, using cell obtained from at least three different donors.

Results

Activation of NF-κB by Amadori adducts in HPMCs

In a first instance, we checked the absence of AGEs in the glycated preparations used in the present study by measuring AGE-related fluorescence at excitation maximum of 370 nm and emission maximum of 440 nm. The fluorescence values obtained in the highly glycated haemoglobin solution (HHb; 10 nM) or the glycated BSA solution (gBSA; 0.25 mg ml^{-1} equivalent to approximately 4 μM) were below the fluorescence values obtained with the 0 mg ml^{-1} concentration of the AGE-BSA in our standard curve (data not shown), therefore confirming the absence of AGEs in the glycated preparations.

We then studied whether Amadori adducts can modify NF-κB activity in HPMCs. EMSA revealed that incubation (1 h) with both 10 nM HHb and 0.25 mg ml^{-1} gBSA significantly induced NF-κB-binding activity as compared with cells treated with normal-glycated haemoglobin (NHb) or nonglycated BSA (nBSA) (Figure 1a). The NF-κB-specific binding band was eliminated by the addition of a 100-fold excess of unlabelled NF-κB oligonucleotide to the reaction mixture (not shown).

Similarly, transiently transfected HPMCs with the p5 × NF-κB-Luc reporter plasmid showed a clear stimulation of NF-κB-dependent transcription by HHb and gBSA, but not by NHb or nBSA, after 12 h exposure (Figure 1b). Increased NF-κB-dependent transcription was observed as early as 6 h and it was sustained for at least 18 h after cell stimulation with HHb (around 1.5-, three-, 4.8-, and four-fold increase over basal

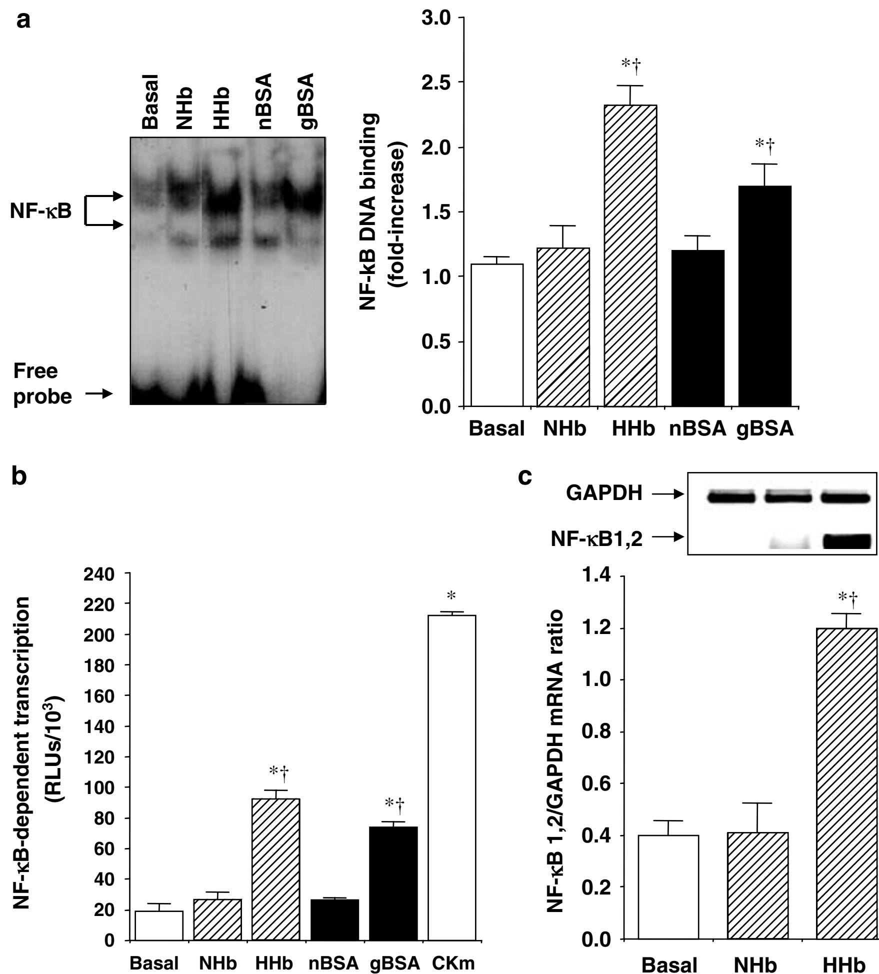

Figure 1 Amadori adducts activate NF-κB in HPMCs. (a) DNA-binding activity of NF-κB was assessed after 1 h treatment of HPMCs with either NHb or HHb (both at 10 nM) or gBSA and nBSA (both at 0.25 mg ml^{-1}). A representative EMSA assay is shown, $n = 9$. (b) NF-κB-dependent transcriptional activation was assessed in cells transiently transfected with p5 × NF-κB-Luc plasmid. HPMCs were exposed for 12 h to the above-described compounds, after which luciferase activity (RLUs) was measured, $n = 32$. (c) Expression of NF-κB1 and NF-κB2 mRNAs after HPMCs exposure to HHb or NHb (both at 10 nM) was analysed by RT–MPCR assay. A representative blot and densitometry values of the ratio mRNA NF-κB1, 2/GAPDH mRNA are shown, $n = 9$. Results are expressed as mean ± s.e.m. $^*P \leqslant 0.05$ vs basal; $^†P \leqslant 0.05$ vs the respective glycosylation control.

at 6 h, 8 h, 12 h, and 18 h, respectively, $P \leqslant 0.05$ by ANOVA) (see Supplemental data, Figure 2). A cytokine mix (CKm) consisting of IL-1β and TNF-α (both at 10 ng ml^{-1}) was used as a positive control for the induction of NF-κB-dependent transcription. We additionally analysed the effect of HHb on mRNA levels of p50/105 (NF-κB1) and p49/100 (NF-κB2) in HPMCs (Figure 1c). The best-characterised form of NF-κB is a heterodimer formed by a 50 kDa (p50/NF-κB1) and a 65 kDa (p65/RealA) protein. RT–MPCR assays revealed an increased band corresponding to NF-κB1 and/or NF-κB2 (143 bp) in HPMCs treated with 10 nM HHb for 6 h, but not with vehicle or 10 nM NHb (Figure 1c) (see also Supplemental data, Figure 3).

Amadori adducts stimulate several NF-κB-related proinflammatory genes in HPMCs

We further studied whether Amadori adducts may affect the expression, activity, and cellular levels of several NF-κB-related proinflammatory markers. Thus, both HHb and gBSA, but not their corresponding glycosylation controls, stimulated the activity (more than three fold increase over basal) of two NF-κB-related proinflammatory enzymes such as NOS and COX (Table 1). HHb (10 nM) also induced a significant increase on the basal secretion of several NF-κB-related proinflammatory cytokines, such as IL-6 (4.9 ± 2.6 vs 1.8 ± 0.2 pg ml^{-1}, $P \leqslant 0.05$), TNF-α (118.0 ± 15.1 vs 55.0 ± 5.5 pg ml^{-1}, $P \leqslant 0.05$), and IL-1β (12.5 ± 3.1 vs 4.3 ± 3.2 pg ml^{-1}, $P \leqslant 0.05$), in cell supernatants. NHb failed to modify the basal production of these cytokines (see also Supplemental data, Figure 4).

We therefore determined the influence of HHb and gBSA on the expression of *NOS*, *COX*, *IL-6*, *TNF-α*, and *IL-1β* genes. As assessed by transient transfection, iNOS promoter activity was significantly increased after exposure for 12 h to both Amadori adducts (Figure 2a). To confirm a possible transcriptional regulation, we tested whether inhibition of RNA synthesis was able to abrogate the increase in iNOS mRNA levels induced by HHb. To this end, we used a long half-life RNA polymerase inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB, 10 μM). After preincubation with DRB for 1 h, HPMCs were exposed to HHb for additional 12 h. HHb-dependent iNOS mRNA upregulation was significantly reduced by DRB treatment, as shown in Figure 2b. DRB also showed a significant blockade of the iNOS promoter activity (3.19 ± 0.3- and 1.16 ± 0.06-fold induction over basal levels for HHb and HHb + DRB, respectively, $P \leqslant 0.05$). In addition, HHb had no effect on endothelial NOS (eNOS) activity and gene expression (around 1.05 ± 0.07-fold induction over basal, $P \leqslant 0.05$) (see Supplemental data, Figure 5a).

Similarly, cell treatment with both Amadori adducts selectively stimulated the activity of a short human *COX-2* promoter construct transiently transfected into HPMCs (Figure 2c), without affecting the activity of the human *COX-1* gene promoter (1.04 ± 0.01- and 0.99 ± 0.2-fold induction over basal for HHb and gBSA, respectively, $P \leqslant 0.05$). Furthermore, exposure to HHb for 6 h induced a selective upregulation of the *COX-2* gene, as determined by RT–MPCR (Figure 2d). Again, no significant effect was observed on *COX-1* mRNA expression upon HHb treatment (1.01 ± 0.03-fold induction over basal, $P \leqslant 0.05$) (see Supplemental data, Figure 5b).

Figure 2e shows that HHb and gBSA significantly enhanced IL-6 promoter activity. Indeed, HHb stimulated IL-6 and IL-6R mRNA expression (Figure 2f, open and solid bars, respectively). Furthermore, HHb also stimulated TNF-α and IL-1β mRNA gene expression (2.9 ± 1- and 3.0 ± 0.28-fold induction of basal, respectively, $P \leqslant 0.05$) and their respective receptors (data not shown).

Finally, to test whether NF-κB transcriptional activation was required for these Amadori-induced effects on proinflammatory molecules, we evaluated the effect of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). As shown in Figure 3a, PDTC (100 μM) prevented the stimulatory effect of both HHb and gBSA on iNOS, COX-2, and IL-6 promoters (Figure 3a). In accordance with these results, PDTC abrogated HHB- and gBSA-related NOS and COX activities (Figure 3b).

Table 1 Effect of Amadori adducts on NOS and COX enzymatic activities in HPMCs

Effector	*NOS activity* (NO_x, μM)	*COX activity* (RLUs /10^3)
None	3.9 ± 2.0	5.7 ± 3.3
NHb	4.0 ± 1.8	4.4 ± 1.1
HHb	15.3 ± 2.5*	26.1 ± 12.1*
HHb + L-NAME	$3.7 \pm 0.3^{\dagger}$	ND
HHb + indomethacin	ND	$12.2 \pm 8.0^{\dagger}$
nBSA	4.2 ± 0.3	5.3 ± 1.2
gBSA	12.3 ± 4.2*	15.7 ± 7.2*
CKm	11.3 ± 2.9*	33.2 ± 3.3*

ND = not determined.
The effectors were used at the following concentrations: HHb and NHb at 10 nM, gBSA and nBSA at 0.25 mg ml^{-1}, L-NAME at 100 μM, and indomethacin at 10 μM, $n = 18$. *$P \leqslant 0.05$ vs basal; $^{\dagger}P \leqslant 0.05$ vs HHb-treated cells. NO_x, nitrate + nitrite.

Participation of ROS on the Amadori-induced effects in HPMCs

As NF-κB is a redox-regulated factor, we also analysed the effect of several ROS scavengers on the proinflammatory effects of Amadori adducts in HPMCs. As shown in Figure 4, pretreatment with superoxide dismutase (SOD, 200 U ml^{-1}), the cell-permeable SOD-mimetic Tempol (100 μM), or dimethylthiourea (DMTU, 1 mM) prevented the stimulatory effect of both HHb and gBSA on NF-κB-dependent transcription and iNOS, COX-2 ($-327/+59$ bp), and IL-6 promoter activation (Figures 4a, b, and d, respectively). The ROS scavengers used did not significantly modify basal transcription levels (data not shown). Tempol also prevented HHb-induced iNOS and COX-2 protein expression, as shown by Western blotting (Figure 4c). Similarly, SOD, Tempol, and DMTU impaired the stimulatory effect of HHb on NOS and COX activities (Table 2). Tempol also prevented the increased secretion of IL6, TNF-α, or IL-1β induced by HHb in HMPCs (Table 2).

NO and peroxynitrite as mediators of HHb-induced NF-κB-dependent transcriptional activation in HPMCs

In order to investigate a potential role for NO in NF-κB activation, we performed transient transfection assays with

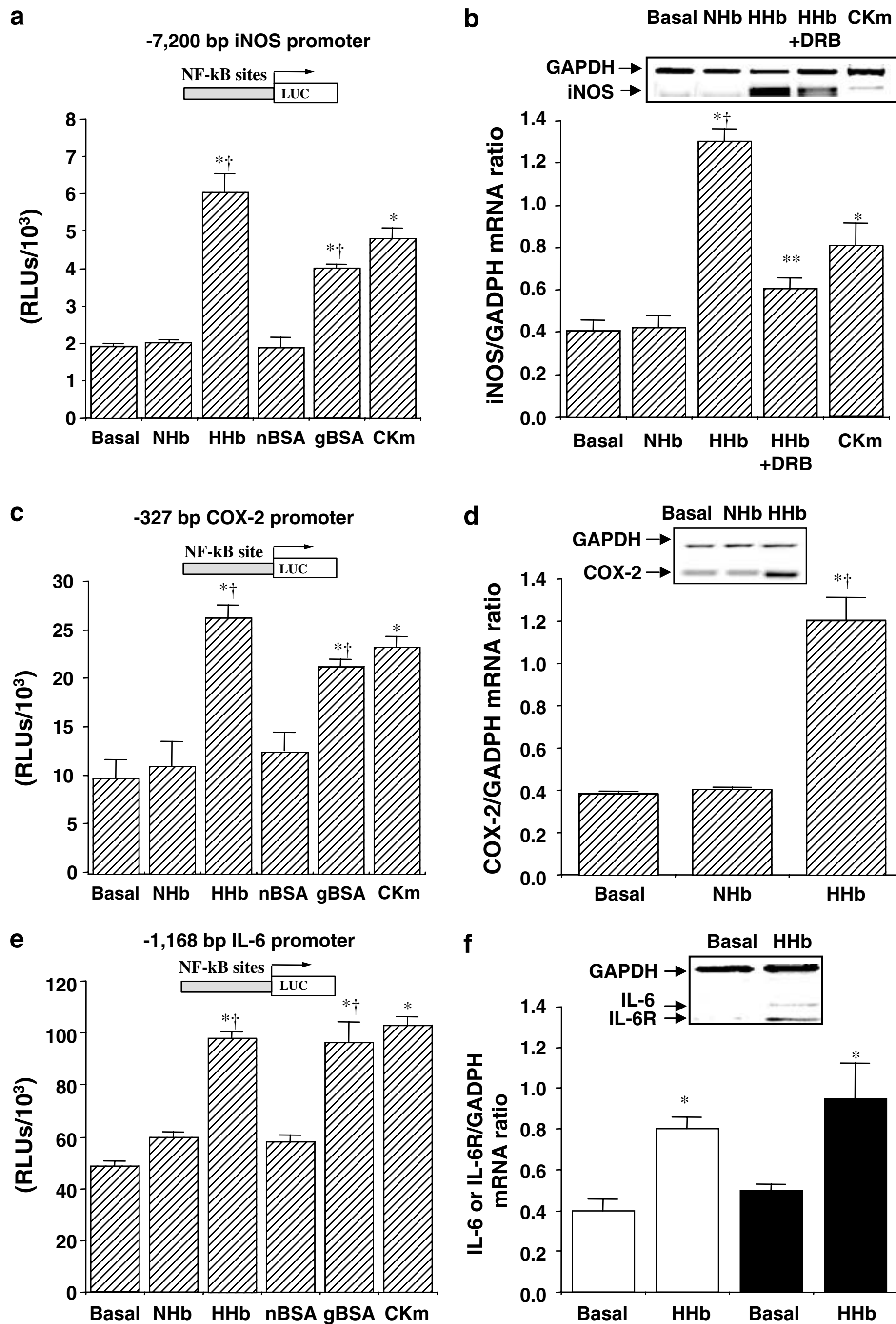

Figure 2 Stimulation by Amadori adducts of NF-κB-related proinflammatory gene expression. The effect of Amadori adducts on either human iNOS (a), human −327/+59 COX-2 (c), and human IL-6 (e) promoters was studied using luciferase-based reporter plasmids in transiently transfected HPMCs. Cells were treated for 12 h with HHb and NHb (both at 10 nM), gBSA, and nBSA (both at 0.25 mg ml^{-1}) or a cytokine mixture (TNF-α + IL-1β, 10 ng ml^{-1} each), $n = 32$. The effect of a 6 h treatment with the above-described compounds on iNOS (b), COX-2 (d), and IL-6 (open bars) and IL-6 receptor (solid bars) (f) mRNA levels was also determined by RT–MPCR assays. Representative blots are shown, $n = 9$. DRB: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (10 μM). Results are expressed as mean ± s.e.m. *$P \leqslant 0.05$ vs basal; †$P \leqslant 0.05$ vs the respective glycosylation control; **$P \leqslant 0.05$ vs HHb-treated cells.

p5 × NF-κB-Luc, in the presence of different iNOS inhibitors, either specific (1400W) or nonspecific (L-NAME). As shown in Figure 5a, both 1400W (10 μM) and L-NAME (100 μM) reduced HHb-stimulated NF-κB activity to an extent similar to that obtained in the presence of the anti-inflammatory glucocorticoid dexamethasone (Dex). In unstimulated HPMCs, NOS inhibitors and Dex failed to modify NF-κB activity by themselves (data not shown). PDTC (100 μM) was used as a control for blocking HHb-induced NF-κB activation (see Supplemental data, Figure 6).

Furthermore, incubation of HPMCs with two exogenous NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP) and

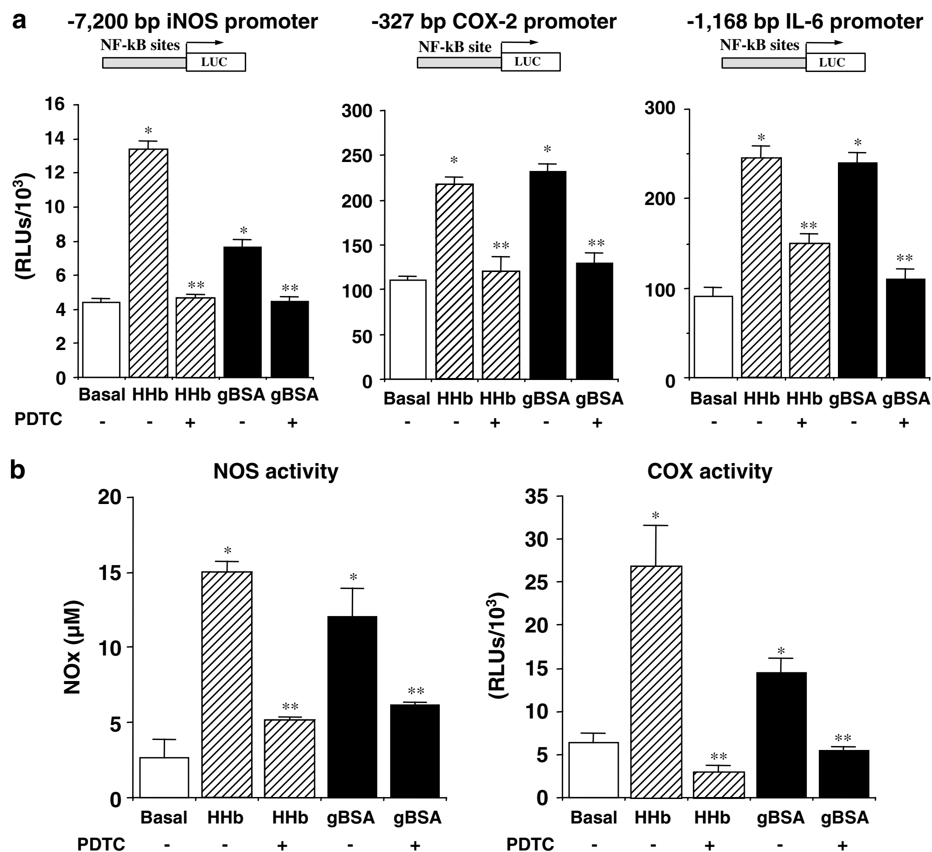

Figure 3 Blockade of Amadori-induced stimulation of NF-κB-related proinflammatory factors by PDTC. (a) HPMCs were treated for 12 h with HHb (10 nM) or gBSA (0.25 mg ml^{-1}) either alone or in combination with PDTC (100 μM) and the luciferase-based expression of human iNOS, −327/+59 human COX-2, and human IL-6 was studied in transiently transfected HPMCs. (b) NOS and COX activities were determined after HPMCs exposure to the above-described treatments, $n = 32$. *$P \leqslant 0.05$ vs basal; **$p \leqslant 0.05$ vs the correspondent Amadori (gBSA or HHb)-treated cells.

sodium nitroprusside (SNP) resulted in the stimulation of this NF-κB-Luc reporter construct (Figure 5b). Both SNAP (10 μM) and SNP (100 μM) showed a cooperative effect with HHb (Figure 5b), but not with NHb (not shown).

A role for peroxynitrite in HHb-induced NF-κB activation was also analysed. Treatment of HPMCs with the peroxynitrite scavenger's uric acid (1 and 10 μM) and Trolox® (1, 5, and 10 μM) partially blocked (40–55%) the stimulation of NF-κB-dependent transcription elicited by HHb (Figure 5c). The partial nitration of different proteins (up to 45%) in HPMCs stimulated by HHb was confirmed by Western blot experiments using a 3-nitrotyrosine antibody (Figure 5d, see arrows). HHb-induced nitration was almost suppressed by coincubation with Tempol, and in a lesser extent by DMTU (Figure 5d).

Discussion

The use of CAPD is increasing as a renal replacement therapy in diabetic patients undergoing dialysis. This therapeutic option has more complications in diabetic patients than in nondiabetic patients (Stein *et al*., 2004). Hyperglycaemia or events linked to hyperglycaemia have risen as potentially culprit mechanisms explaining this poor outcome (Nakamoto *et al*., 2002; Stoenoiu *et al*., 2002). Hyperglycaemia is now clearly identified as a pivotal factor in other diabetes-associated complications, including the vascular ones. Among the mechanisms by which hyperglycaemia can contribute to diabetic vasculopathy, we and others have highlighted in previous works a role for early products of nonenzymatic protein glycosylation, the so-called Amadori adducts, in promoting NO-related endothelial dysfunction and inflammation in the vascular wall (Angulo *et al*., 1996; Amore *et al*., 1997; Peiró *et al*., 1998; Vallejo *et al*., 2000a; Peiró *et al*., 2001; Hattori *et al*., 2002; Peiró *et al*., 2003; Rodríguez-Mañas *et al*., 2003). We therefore aimed to analyse whether Amadori adducts may play a role in hyperglycaemia-associated PM dysfunction. We focused on the effects of Amadori adducts on mesothelial cells, which share a common embryological derivation with vascular endothelial cells (Hernando *et al*., 1994), and have been proposed to be on the basis of peritoneal dysfunction (Yañez-Mo *et al*., 2003; Yao *et al*., 2003) through the production of different growth factors and proinflammatory markers (Riese *et al*., 1999; Mandl-Weber *et al*., 2002).

We observed a significant stimulation of NF-κB in HPMCs treated with Amadori adducts. As a result of this, increased transcriptional activity of different proinflammatory genes, such as *IL-6*, *TNF-α*, *IL-1β*, *iNOS*, and *COX-2*, and increased

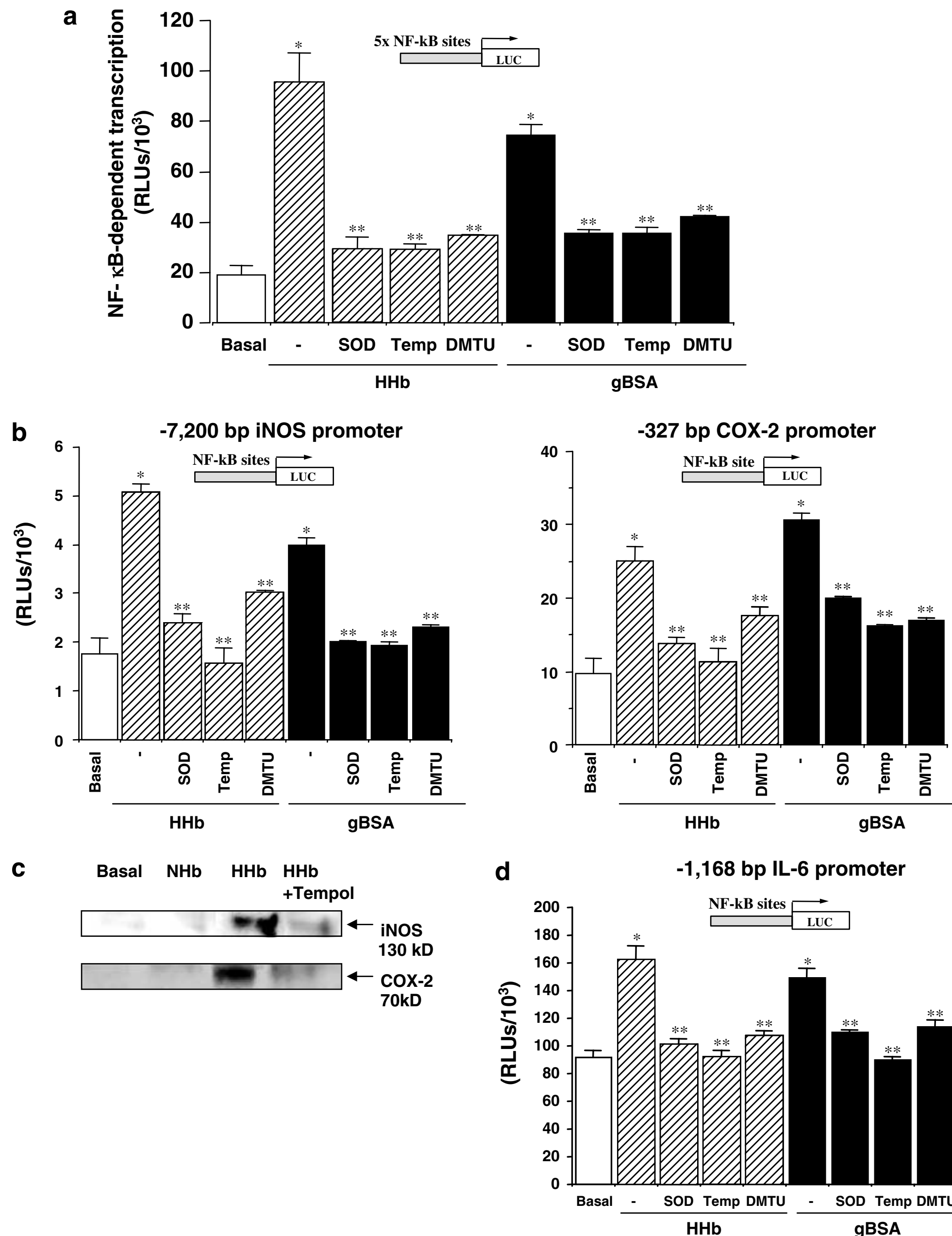

Figure 4 Participation of ROS in the proinflammatory effects induced by Amadori adducts in HPMCs. (a) Transient transfection experiments were performed to measure NF-κB-dependent transcription activity after 12 h exposure to HHb (10 nM) or gBSA (0.25 mg ml^{-1}) either alone or co-incubated with superoxide dismutase (SOD 200 U ml^{-1}), Tempol (Temp, 100 μM) or dimethylthiourea (DMTU, 1 mM), $n = 18$. Under the same conditions, human iNOS and −327/+59 human COX-2 (b), as well as human IL-6 (d) promoters luciferase-based reporter activities were assessed by transient transfection assays, $n = 18$. (c) A representative immunoblot for iNOS and COX-2 protein is shown after 12 h exposure of HPMCs to HHb either alone or in combination with Tempol at the above-described concentrations, $n = 9$. *$P \leqslant 0.05$ vs basal. **$P \leqslant 0.05$ vs the correspondent Amadori (gBSA or HHb)-treated cells.

levels of their resulting proinflammatory proteins were observed. This result is consistent with previous reports demonstrating their ability to stimulate NF-κB itself or other inflammation-related transcription factors, like activator protein 1 (AP-1), in vascular smooth muscle cells (Hattori *et al*., 2002; Peiró *et al*., 2003).

It is worth noting that both haemoglobin and albumin were used at concentrations that can be found in the circulation under physiological conditions (Tietz, 1990). Glycated albumin has been used in previous reports as a model for Amadori adducts, based on the close correlation found between serum glucose content and the degree of albumin glycation. However, in the experimental approach performed in our laboratory, glycated haemoglobin has been preferentially chosen as a model of circulating Amadori adduct because of the following reasons: (i) haemoglobin is very sensitive to changes in

Table 2 Effects of antioxidants on NOS and COX activities, and proinflammatory cytokine levels induced by HHb in HPMCs

Effector	*NOS activity* (NO_x, μM)	*COX activity* (RLUs/10^3)	*IL-6* (pg ml^{-1})	*TNF-α* (pg ml^{-1})	*IL-1β* (pg ml^{-1})
None	3.8±2.1	7.5±2.4	1.9±0.2	53.9±6.2	4.3±2.8
HHb	14.0±1.8*	26.9±11.1*	5.8±2.6*	120.0±4.0*	12.3±3.6*
HHb+SOD	4.9±0.1†	16.7±3.8†	ND	ND	ND
HHb+Tempol	5.3±0.3†	10.8±1.1†	2.8±0.1†	81.0±10.1†	6.0±1.0†
HHb+DMTU	3.7±0.1†	11.6±5.8†	ND	ND	ND

ND = not determined.
The effectors were used at the following concentrations: HHb and NHb at 10 nM, SOD at 200 U ml^{-1}, Tempol at 100 μM, and DMTU at 1 mM, $n=9$. *$P \leqslant 0.05$ vs basal; †$P \leqslant 0.05$ vs HHb-treated cells.

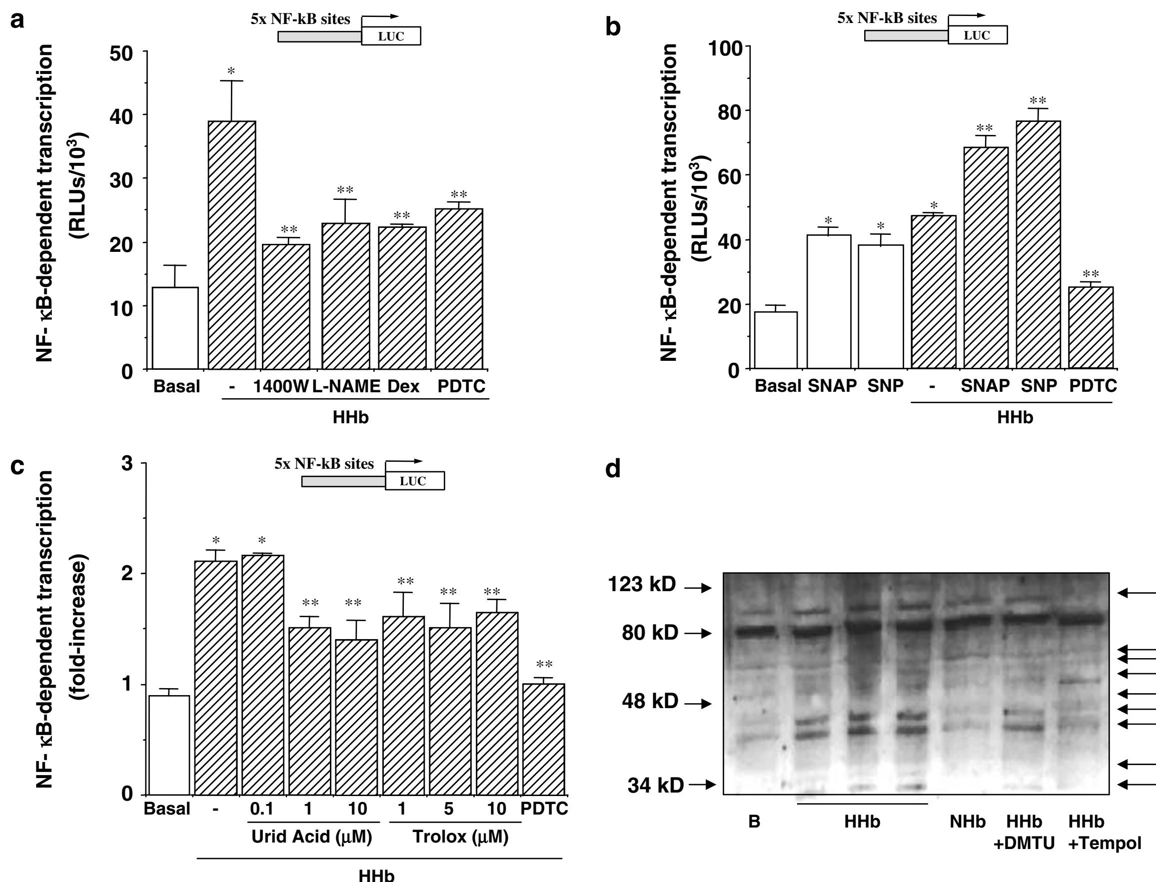

Figure 5 NO and peroxynitrite modulate NF-κB-dependent transcriptional activity in HPMCs. (a) HPMCs transiently transfected with pNF-κB-Luc were treated with HHb (10 nM) alone or in combination with the iNOS inhibitors 1400W (10 μM) and L-NAME (100 μM), dexamethasone (Dex, 1 μM) or PDTC (100 μM) for 12 h, after which luciferase activity was measured, $n=12$. (b) NF-κB-dependent transcription was further determined in HPMCs treated for the same time period with *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10 μM) and sodium nitroprusside (SNP 100 μM) either alone or in the presence of HHb. HHb was also coincubated with PDTC (100 μM), $n=9$. (c) NF-κB-dependent transcription was determined in HPMCs treated for 8 h with HHb alone or in combination with the peroxynitrite scavengers uric acid (0.1, 1, and 10 μM) and Trolox (1, 5, and 10 μM) or PDTC (100 μM), $n=9$. (d) Representative immunoblot for nitrated protein expressions after exposure for 12 h to either NHb or HHb (both at 10 nM) or HHb in combination with Tempol or DMTU (100 μM and 1 mM, respectively).*$P \leqslant 0.05$ vs basal; **$P \leqslant 0.05$ vs HHb-treated cells.

glycaemic concentrations (Jovanovic & Peterson, 1981); (ii) it circulates free in plasma at nanomolar concentrations (Tietz, 1990); and (iii) it can penetrate into the vascular tissue in nonpathological circumstances (Paredi *et al*., 1999). Actually, there is evidence indicating that circulating proteins, including free haemoglobin, can be incorporated into tissues either by a transcytosis mechanism, as it has been shown in mesothelial cells (Bodega *et al*., 2002), or by an active transmembrane transport in other cell types (Wu & Cohen, 1994). The existence of nonenzymatic protein glycosylation has been also reported during CAPD in the mesothelial layer of the human peritoneum (Posthuma *et al*., 2001). In addition, *in vivo* and

in vitro kinetics data provide evidence for the formation of early-glycated proteins in the peritoneal cavity during the time course (10 h) of the routine peritoneal equilibration test (Friedlander *et al*., 1996). It has also been suggested that glycated proteins can move from plasma into the peritoneal cavity (Friedlander *et al*., 1996).

Although Amadori adducts have been described as important precursors of AGEs (Makita *et al*., 1992), the proinflammatory effects of the glycated solutions on HPMCs shown in this study can be attributed to Amadori adducts, as the presence of detectable AGEs in the solutions was discarded. Thus, this seems to be a separate mechanism from that induced by AGEs (Boulanger *et al*., 2002; Wu *et al*., 2002; Rashid *et al*., 2004), as inferred by several recent studies (Mandl-Weber *et al*., 2001; Hattori *et al*., 2002; Valencia *et al*., 2004). In this regard, a possible role for metal ions or endotoxin contamination was discarded mainly by the ability of Amadori proteins to release ROS even in the presence of EDTA (Vallejo *et al*., 2000b), or by a detection kit (see Methods), respectively.

The ability of Amadori adducts for inducing proinflammatory factors in HPMC cultures appears to be mediated by different ROS. These results are consistent with previous reports showing the ability of Amadori adducts to produce ROS, mainly superoxide anions (Vallejo *et al*., 2000b; Yoo *et al*., 2004). In this way, it is well accepted that increased oxidative stress plays a key role in the development of vascular inflammation and vasculopathy in diabetes (Spitaler & Graier, 2002). It seems therefore reasonable to propose an analogous role for ROS in the putative alterations of peritoneum membrane functionality associated to hyperglycaemia.

Concerning a role for NO in PM dysfunction, it is thought to be involved in both structural and permeability alterations (Chen *et al*., 2000; Mandl-Weber *et al*., 2002). Currently, a fact widely debated is the definitive source of local NO at the peritoneum. Our data agree with previous reports showing a barely detectable basal iNOS activity, (Chen *et al*., 2000; Davenport *et al*., 2004). However, upon treatment with Amadori adducts, a clear stimulation of iNOS activity and gene expression was observed. Thus we can conclude that, at present, several extracellular stimuli are known to induce iNOS in HPMCs, including cytokines (this work, and Chen *et al*., 2000), the insoluble polysaccharide zymosan, which acts as a peritonitis inducer (Yao *et al*., 2004), or, as shown in the present study, Amadori adducts. Although eNOS stimulation has also been involved in NO production by mesothelial cells (Reimann *et al*., 2004), Amadori adducts did not stimulate eNOS activity in HPMCs.

Finally, our results suggest a possible role for Amadori-induced NO or NO-derived compounds in peritoneal complications related to hyperglycaemia through the activation of NF-κB in HPMCs. This is in accordance with the fact that NO and NO-derived compounds have emerged in the last years as important modulators of gene expression through their ability to modulate several transcription factors (Liaudet *et al*., 2000; Cooke & Davidge, 2002). Indeed, modulation of NF-κB activity by NO appears to have an important role in the regulation of the inflammatory response (Liaudet *et al*., 2000). Nevertheless, in a pro-oxidant environment, NO can react with superoxide anions leading to peroxynitrite production, which may in turn further stimulate NF-κB-dependent transcription in HPMCs. Our results indicate a role for peroxynitrite as a mediator for NF-κB activation**,** in HPMCs, as previously proposed in vascular cells (Cooke & Davidge, 2002; Hattori *et al*., 2004). However, the fact that a certain degree of NF-κB-dependent transcription still occurred in the presence of peroxynitrite scavengers discards peroxynitrite as the sole mediator of NF-κB activation by glycated haemoglobin. At present, little is known about the cellular signalling pathways activated by NO and peroxynitrite in HPMCs.

In conclusion, we propose that Amadori adducts can alter mesothelial cell functionality by increasing oxidative and nitrosative stress and by activating NF-κB-related proinflammatory pathway, which may be on the basis of a low-grade proinflammatory response within the peritoneum.

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