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RESEARCH PAPER

Pro-inflammatory effects of early non-enzymatic glycated proteins in human mesothelial cells vary with cell donor's age

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Background and purpose: Diabetes mellitus is prevalent in the elderly population. It is also a disease causing tissue damage through several different mechanisms. Some of these mechanisms are also activated by ageing and this overlap raises questions about how diabetes induces damage in the elderly. Early products of non-enzymatic glycation of proteins (Amadori adducts), and the ageing process share the capacity to induce oxidative stress and inflammation in human peritoneal mesothelial cells (HPMCs). We have evaluated the interactions between the age of the donor of the HPMCs and the pro-inflammatory effects of Amadori adducts in those cells.
Experimental approach: HPMCs were isolated from 20 individuals (age range 21–81 years) and grown in culture. Using different experimental approaches we determined NF-κB dependent transcriptional activity and different NF-κB-related pro-inflammatory gene and protein expressions in basal (or non-stimulated) conditions and after stimulation with two Amadori adducts; highly-glycated haemoglobin and glycated bovine serum albumin.
Key results: Amadori-induced effects on NF-κB dependent-transcription and on the activity of NOS, COX and several NF-κB-related pro-inflammatory genes (iNOS, COX-2, TNF-α, IL-1β, and IL6) diminished as the donor's age increased, being practically absent in cells from donors more than 65 years old. Such decreased effects were inversely correlated with an increased basal expression and activity of these pro-inflammatory markers with age.
Conclusions and implications: Pro-inflammatory effects of Amadori-adducts in HPMCs were strongly dependent on cell donor's age. This may have significant implications for the mechanisms underlying diabetes-induced tissue damage in patients of different ages.
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Keywords: Amadori adducts; ageing; diabetes; glycated human haemoglobin; human mesothelial cells; inflammatory response; nitric oxide; nuclear factor-κB

Abbreviations: AGEs, advanced glycation end products; CAPD, continuous ambulatory peritoneal dialysis; COX, cyclooxygenase; gBSA, glycated bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HHb, highly glycated oxyhaemoglobin; HPMCs, human peritoneal mesothelial cells; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; NHb, oxyhaemoglobin glycated at normal levels; PBS, phosphate-buffered saline; ROS, reactive oxygen species

Introduction

Among the substances involved in tissue damage induced by diabetes mellitus, Amadori adducts have been shown to play a significant role (Angulo *et al*., 1996; Amore *et al*., 1997; Mandl-Weber *et al*., 2001; Hattori *et al*., 2002; Rodríguez-Mañas *et al*., 2003). Particularly, human haemoglobin, when highly glycated (HHb) has been shown to induce functional and structural changes by means of reactive oxygen species (ROS) and inflammation in several systems and tissues (Peiró *et al*., 1998; Vallejo *et al*., 2000; Peiró *et al*., 2003; Nevado *et al*., 2005).

We have recently shown that early glycated proteins, also called Amadori adducts, such as HHb and glycated bovine serum albumin (gBSA) may favour a proinflammatory state in human peritoneal mesothelial cells (HPMCs). Such a

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proinflammatory effect, which involves ROS generation, is owing to an exacerbated activity of the nitric oxide synthase (NOS) pathway with enhanced expression of several nuclear factor-κB (NF-κB)-related proinflammatory genes (Nevado *et al*., 2005).

Around 20% of people more than 65 years old have diabetes mellitus, with important consequences on their total and active life expectancy (Maggi *et al*., 2004). Different authors have reported that advancing age is accompanied by a basal prooxidative and proinflammatory state (Ferrucci *et al*., 2004), similar to that induced by diabetes or Amadori adducts. Such an observation has been made in many systems derived from old animals, including human beings (Sarkar *et al*., 2004) and different cell types, including mesothelial cells (Nevado *et al*., 2006). This fact raises the question of whether substances with pro-oxidant and proinflammatory properties, like Amadori adducts, still exert the same effect in situations characterized by a significant basal proinflammatory state, as it happens during the ageing process, or whether the effect of such substances is blunted or attenuated in such basal-enhanced proinflammatory situations. The age of the patient may thus arise as a modulating factor in the patho-physiology of the damage induced by diabetes in some tissues, including among many others the changes observed in the peritoneum during peritoneal dialysis, where inflammatory factors seem to play a role in trans-differentiation of mesothelial cells (Yañez-Mo *et al*., 2003). Therefore, we aimed to study the effect of ageing on the low-grade inflammatory response elicited by Amadori adducts in human cultured omental mesothelial cells obtained from patients of different ages. Our data showed that proinflammatory effects induced by Amadori adducts in HPMCs depend on the age of the donor of the HPMCs.

Methods

Cell culture

The study was approved by the Clinical Research and Ethics Committee of Hospital Universitario de Getafe (CEIC-Área10 no. 04/39, 15 June 2004). HPMCs were isolated from omental tissue from 20 persons (see supplementary data, Table 1) undergoing non-urgent, non-septic abdominal surgery, using a previously described method (Chung-Welch *et al*., 1997; Nevado *et al*., 2005, 2006). Cell cultures between passages two and five were used. The morphologic and immunofluorescence-staining features (Nevado *et al*., 2005, 2006, and supplementary data, Table 2) of the cells remain stable during these passages.

Preparation of Amadori adducts

Lyophilized human haemoglobins, non-enzymatically glycated 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 (St Louis, MO, USA). Before use, haemoglobins were prepared as described previously (Peiró *et al*., 2003; Nevado *et al*., 2005). Briefly, haemoglobins were dissolved in deionized 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 deionized water containing 10 mg ml^{-1} ethylenediaminetetraacetic acid (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 BSA (Sigma Chemical Co.) was glycated by incubation in phosphate-buffered saline (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. Glycation of serum albumin was verified using the thiobarbituric acid assay (Ney *et al*., 1981).

The absence of advanced glycation end products (AGEs) in our glycated preparation 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 (Sell and Monnier, 1989). A standard curve ($R^2 = 0.99$) was carried out using AGE-modified BSA (0.5–5 μg ml^{-1}). The fluorescence values obtained in either the HHb (10 nM) or the gBSA solution (0.25 mg ml^{-1}) were under the fluorescence values obtained with the 0 mg/ml concentration of the AGE–BSA in our standard curve (data not shown). In addition, detection of non-fluorescent AGEs in the glycated oxyhaemoglobin preparations was performed by enzyme-linked immunosorbent assay (ELISA), with the use of a rabbit polyclonal antibody to AGE (Abcam, Cambridge, UK). A standard curve ($R^2 = 0.98$) was carried out using AGE-modified BSA (0–100 μg ml^{-1}). The absorbance values obtained in the HHb or normal glycated haemoglobin solution (NHb) (both at 10 nM) were not significantly different from the absorbance values obtained with the 0 mg/ml concentration of the AGE-modified BSA (0.748 ± 0.021, 0.750 ± 0.020 and 0.740 ± 0.020 arbitrary units for HHb, NHb and AGE-modified BSA, respectively).

Finally, 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).

Reporter plasmids

The reporter plasmids: p5xNF-κB-Luc (Stratagene, La Jolla, CA, USA), human inducible NOS (iNOS) (p7.2 hiNOS-Luc) (Taylor *et al*., 1998) and a short human cyclooxygenase (COX)-2 (phPES2 −327/+59-Luc) (Inoue *et al*., 1995), were used.

Transient transfection and luciferase assays

Transient transfection experiments were performed as we have described previously (Peiró *et al*., 2003). Briefly, HPMC (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 Dulbecco's modified Eagle's medium 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 $\times$, Promega, Madison, WI, USA), 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, USA). Luciferase activity was expressed as relative luciferase units (RLUs, Peiró *et al*., 2003).

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

Measurement of nitrate plus nitrite
Nitrite plus nitrate measurements were performed as we described previously (Nevado *et al*., 2005, 2006). Briefly, 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 a nitric oxide detector (NOA 280 analyzer, Sievers, Boulder, CO, USA). A standard curve was generated by injections of known concentrations of sodium nitrate. The levels of NO_x were normalized to protein content (1 μg).

Measurement of COX activity
Total COX activity was performed as we described previously (Nevado *et al*., 2005, 2006). Briefly, 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, USA) using a specific chemiluminescent substrate to detect the peroxidative activity of COX enzymes in protein extract homogenates, as specified by the manufacturers. Light emission is directly proportional to COX activity in the sample. Results are expressed as RLUs normalized to protein content (1 μg).

RNA isolation and RT–Multiplex PCR assays
Total RNA from HPMC (10^5–10^6 cells) was obtained using RNAquous kit (Ambion Inc., Austin, TX, USA), following the manufacturer's instructions. RT and Multiplex-PCR (MPCR) were performed with appropriate kits (Maxim Biotech Inc., San Francisco, CA, USA), using one 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 glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (hNOSG-MPCR). Levels of mRNA were normalized to GAPDH transcript.

Statistical analysis
Results are expressed as mean $\pm$ s.e.m. as fold induction of the basal (non-stimulated) conditions. Statistical significance ($P<0.05$) using the StatView statistics program (Abacus Concepts Inc., Berkeley, CA, USA) was evaluated using the unpaired Student *t*-test or factorial analysis of variance, as required. A simple regression analysis was carried out to establish the potential correlation between age and each variable of interest. The '*n*' denotes the number of experiments performed in triplicates, using cells obtained from at least three different donors for each group (<65 years, adult cells (AC) or $\geqslant$65 years, old cells (OC)).

Materials
M199 medium, L-glutamine and streptomycin/penicillin solutions were purchased from Biochrom KG (Berlin, Germany), whereas culture plasticware was from Corning-Costar (New York, NY, USA). PBS, foetal calf serum and trypsin-EDTA were purchased from Amresco (Solon, OH, USA), Biological Industries (Beit-Hamek, Israel), and GIBCO BRM (Paisley, UK), respectively. Human TNF-α and IL-1β were from Peprotech (London, UK) and R&D systems (Minneapolis, MN, USA), respectively. *Taq* DNA polymerase and deoxynucleoside triphosphates from ECOGEN (Barcelona, Spain). Unless otherwise stated, all other reagents were purchased from Sigma Chemical Co.

Results

Influence of age on Amadori-induced NOS and COX activities
Age modulated the effects elicited by HHb (10 nM) on two critical enzymes in the inflammatory processes, NOS and COX in HPMCs. Indeed, the effect of HHb treatment (12 h) on levels of nitrate plus nitrite (NO_x) was nearly three-fold lower in cells from the elderly, over 65 years (OC; mean age: 77.00 $\pm$ 3.40 years) than in those from the younger subjects under 65 years (AC; mean age: 42.14 $\pm$ 12.80 years) (Figure 1a). Similar results were obtained with another Amadori adduct; gBSA (0.25 mg ml^{-1}), used to show that this stimulation was not an effect unique to HHb (Figure 1a).

In contrast to the effect of Amadori adducts, a cytokine mixture consisting of IL-1β plus TNF-α (both at 10 ng ml^{-1} each) and used as a positive control for induction of NOS, showed minimal differences among the two age groups (Figure 1a). A clear inverse correlation found between donor's age and the effects of HHb on NOS activity was also shown in Figure 1b.

Furthermore, Figure 1c shows that, mesothelial cell donor's age significantly affected the effect of HHb (10 nM) and gBSA (0.25 mg ml^{-1}) on total COX activity as well. A clear inverse correlation was also found between donor's age and the effects of HHb on COX activity (Figure 1d).

Influence of age on iNOS *and* COX-2 *gene expression, and NF-κB-dependent transcription induced by Amadori adducts*
The ratio of iNOS/GAPDH mRNAs in HPMC treated with 10 nM HHb was significantly lower in OC as compared with

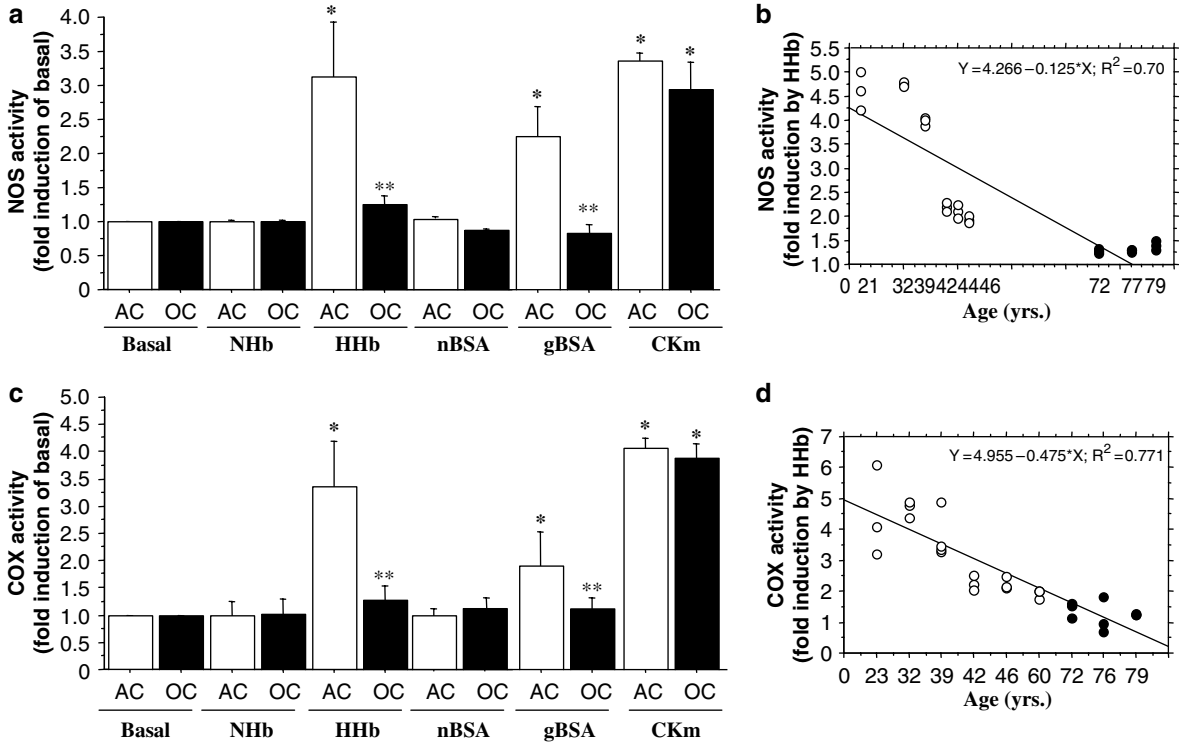

Figure 1 Influence of age on Amadori-induced NOS and COX enzymatic activities in HPMCs. (**a**) Nitrate plus nitrite (NO_x) measurements in cell culture supernatants or (**c**) total COX activity (RLUs) in whole protein extracts were assessed in HPMC from adult young subjects (AC) and old subjects (OC) after 12 h treatment with either NHb, HHb (both at 10 nM); nBSA, gBSA (both at 0.25 mg ml^{-1}) or the cytokine mixture (CKm; IL-1β + TNF-α, 10 ng ml^{-1} each), $n=4$. The correlations between cell donor age and the effects promoted by HHb on either NO_x levels is shown in (**b**) or on total COX activity, shown in **(d)**. *$P\leqslant 0.05$ vs basal, **$P\leqslant 0.05$ vs AC.

AC (Figure 2a). An inverse correlation was also found between donor's age and *iNOS* gene expression (Figure 2b), whereas NHb (10 mM) failed to induce significantly *iNOS* gene expression in either OC or AC (Figure 2a). As found for *iNOS*, *COX-2* gene expression induced by HHb, measured as the COX-2/GAPDH mRNAs ratio was also significantly lower in OC compared to AC (1.3 ± 0.03- vs 2.3 ± 0.58-fold increase over basal in OC and AC, respectively; $P\leqslant 0.0001$, data not shown).

We next transiently transfected HPMCs, obtained from donors of different ages, with either iNOS or a short COX-2 gene promoter reporter plasmid (p7.2 hiNOS-Luc and phPES2 −327/+59-Luc, respectively) and then treated with HHb, NHb or the cytokine mixture (IL-1β + TNF-α), as described above. Figure 2c and e shows that the effects of HHb on both iNOS and COX-2 promoter activities were higher in AC compared to OC. Again, an inverse correlation was found between donor age and the effects of HHb on human iNOS or human COX-2 promoter activities (Figure 2d and f).

We further monitored the effect of age on HHb-induced NF-κB-dependent transcription. HPMC were transiently transfected with p5xNF-κB-Luc reporter plasmid. Again, NF-κB-induced-dependent transcriptional activity by HHb was significantly impaired in OC as compared with AC (Figure 2g). Additionally, a significant inverse correlation was observed between donor age and NF-κB-dependent transcriptional activity induced by HHb (Figure 2h).

When OC and AC were exposed to gBSA (0.25 mg ml^{-1}), similar results to those induced by HHb were obtained, although to a lesser extent. Thus, human iNOS promoter activity was increased (Figure 2c), whereas COX-2 promoter activity showed a comparable increase over basal in OC and AC (Figure 2e). Similarly, NF-κB-dependent transcription was increased (Figure 2g).

However, when HPMC was exposed to the cytokine mixture, no significant differences were observed between OC and AC, regarding human iNOS, and human COX-2 promoter activities or NF-κB-dependent transcriptional activity (Figure 2c, e and g, respectively).

Age-associated effects on HHb-induced proinflammatory cytokine release

The effect of Amadori adducts on the release of three proinflammatory cytokines (IL-1β, IL-6 or TNF-α) by HPMC was evaluated using our most potent stimuli, HHb. Figure 3a shows that HHb (10 nM) enhanced IL-1β, IL-6 or TNF-α levels in HPMC supernatants, this effect being significantly higher in AC than in OC, where it was barely detected. The effects elicited by HHb on the levels of these three cytokines decreased according to the donor's age following a linear regression curve, especially for IL-1β, but also for IL-6 and TNF-α (Figure 3b). On the contrary, NHb (10 nM) did not affect the levels of these cytokines, and age did not modify this pattern at all (Figure 3a).

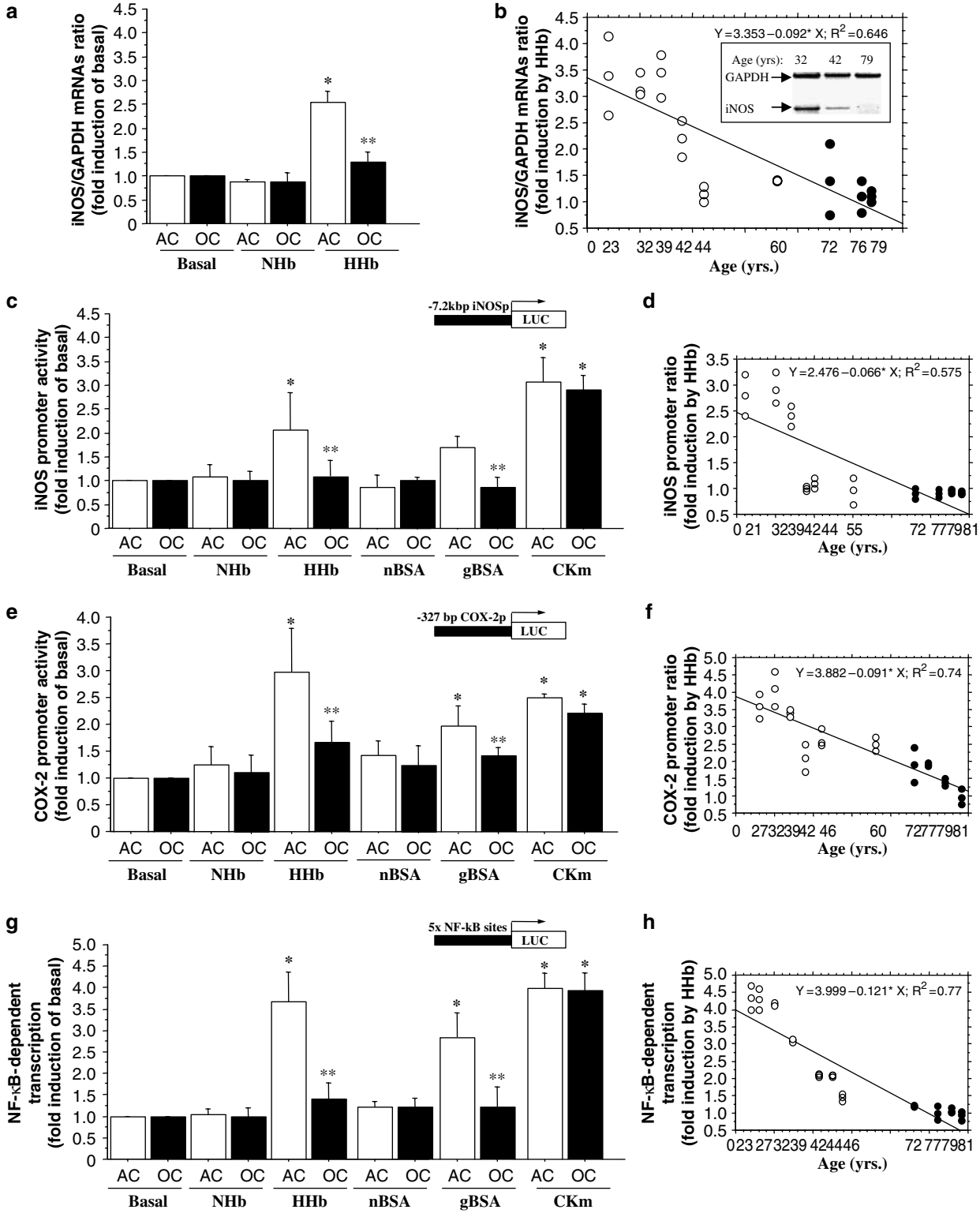

Figure 2 Influence of age on *iNOS* and *COX-2* gene expression, and NF-κB-dependent transcription induced by Amadori adducts in HPMCs. (**a**) Expression of iNOS mRNA after HPMC (from AC; adult subjects, and OC; old subjects) exposure for 12 h to either HHb or NHb (both at 10 nM) was analysed by RT–MPCR assay, ($n=3$). (**b**) A representative blot of the ratio iNOS/GADPH mRNA at different ages was shown. The effect of Amadori adducts on either human iNOS (**c**) or COX-2 (**e**) promoter activity was studied using luciferase reporter plasmids (2 μg each, $n=4$, and $n=5$, respectively) in transiently transfected HPMCs (from AC and OC), and then treated for 12 h with either HHb, NHb (both at 10 nM); nBSA, gBSA (both at 0.25 mg ml^{-1}) or the cytokine mixture (CKm; (IL-1β + TNF-α, 10 ng ml^{-1}, each), after which luciferase activity (as RLUs) was measured. (**g**) NF-κB-dependent transcriptional activity was assessed in cells (from AC and OC) transiently transfected with p5 × NF-κB-luc (1 μg) and then treated for 12 h with the compounds described above after which luciferase activity was measured ($n=5$). Correlations between cell donor age and the effects promoted by HHb on either iNOS/GAPDH mRNAs ratio (**b**), or iNOS promoter activity (**d**) or COX-2 (−327/+59) promoter activity (**f**) or NF-κB-dependent transcriptional activity (**h**). *$P\leqslant 0.05$ vs basal, **$P\leqslant 0.05$ vs AC).

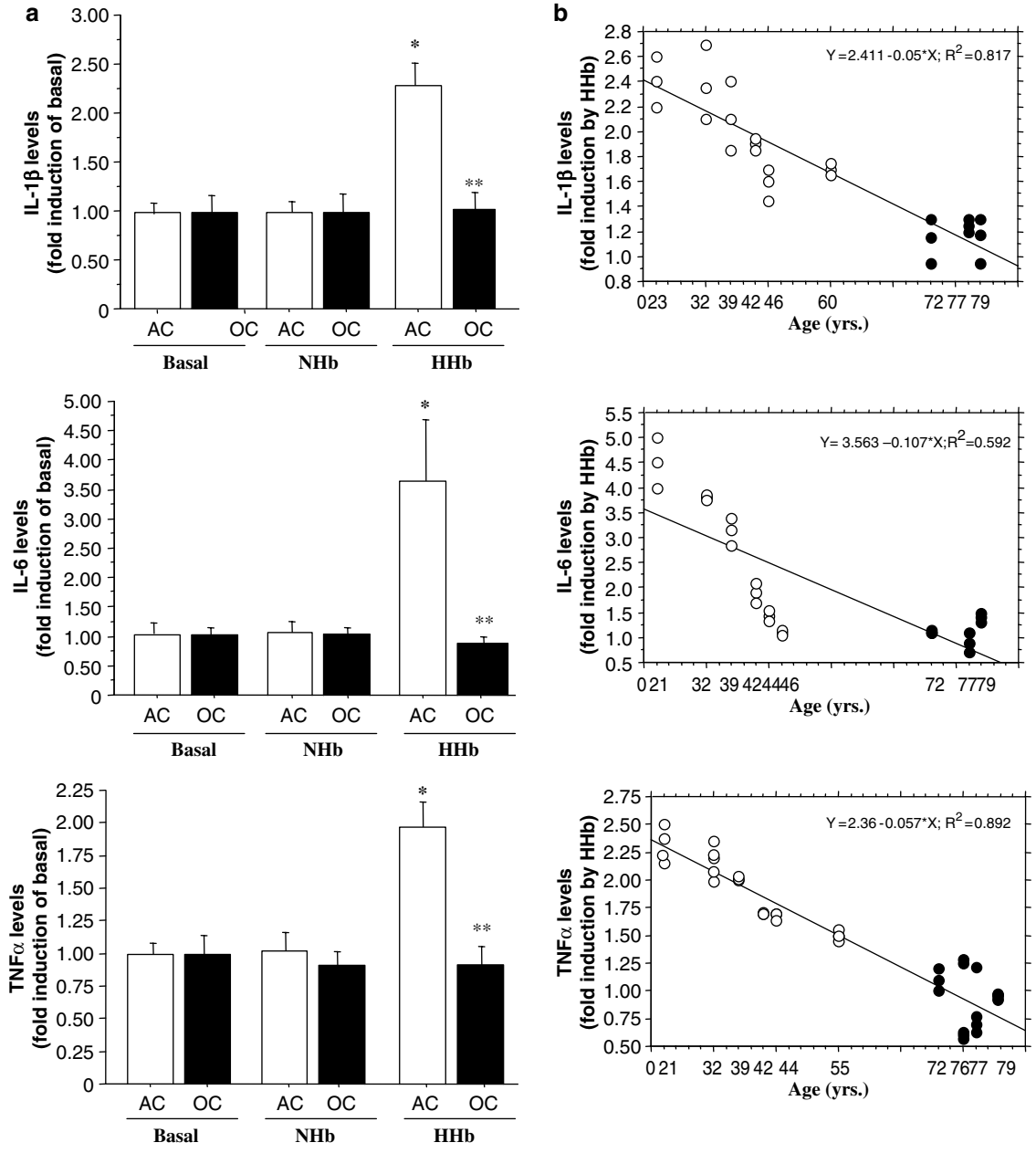

Figure 3 Influence of age on HHb-induced proinflammatory cytokine release in HPMCs. (**a**) Levels of IL-1β, IL-6 and TNF-α were determined by an ELISA-based method in cell culture supernatants of HPMCs (from AC; adult subjects, and OC; old subjects) treated for 12 h with either NHb or HHb (both at 10 nM), $n=4$. (**b**) Correlation between cell donor's age and HHb-induced levels of IL-1β, IL-6 and TNF-α, respectively. *$P\leqslant 0.05$ vs basal, **$P\leqslant 0.05$ vs AC.

Correlation between basal and HHb-induced proinflammatory markers over age

We further compared the levels and activities of several proinflammatory mediators released and expressed by HPMC, relative to donor age, either in basal (non-stimulated conditions) or after stimulation with HHb (and normalized over its respective basal). To achieve this, NF-κB-dependent transcriptional activity and several NF-κB-related proinflammatory markers were measured in both basal and HHb-stimulated cells obtained from subjects of different ages. Thus, as age advances, a clear and significant decrease of HHb-induced effects on NF-κB-dependent transcription (expressed as fold increase over its respective basal) was observed, which was paralleled by an increase in basal NF-κB-dependent transcriptional activity (expressed as RLUs) (Figure 4a).

The same responses were also observed for several NF-κB-related proinflammatory markers, such as iNOS promoter activity, iNOS mRNA levels and therefore for NO_x levels (Figure 4b–d, respectively) or other proinflammatory mediators, such as total COX activity, COX-2 promoter activity and supernatant levels of IL6, IL-1β and TNF-α (Figure 5a–e, respectively). Thus, ageing induces a significant decrease of HHb-induced effects on above-described proinflammatory

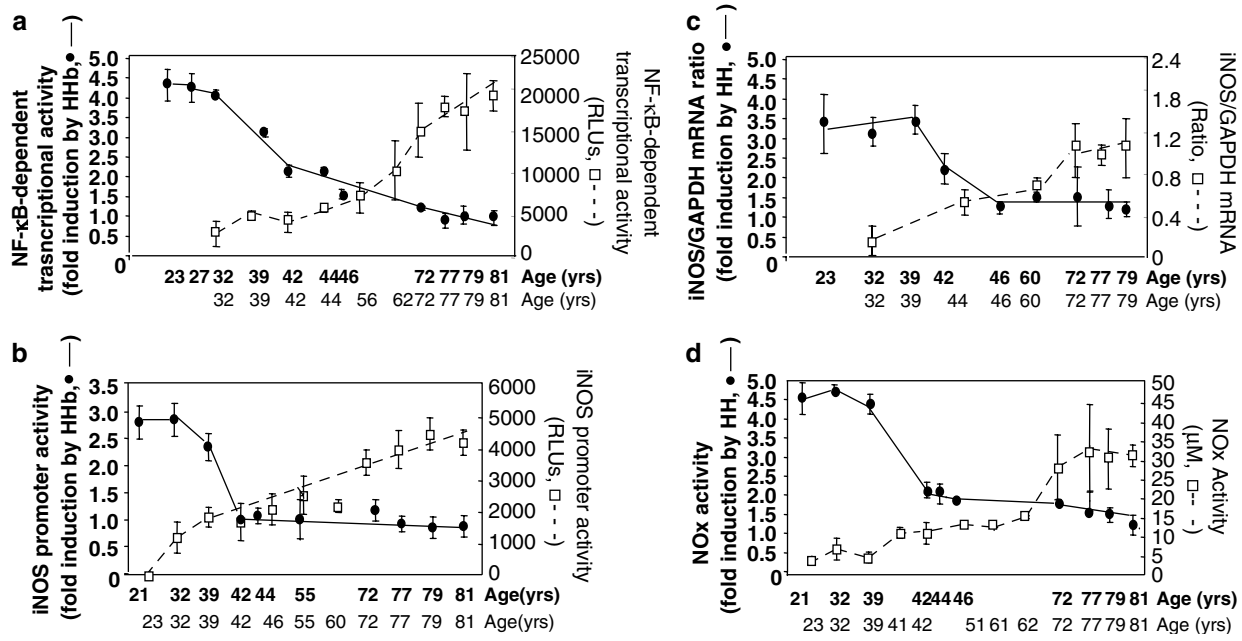

Figure 4 Correlations between basal levels of several proinflammatory markers and HHB-induced effects on the same proinflammatory markers with age (part I). Activities of several proinflammatory mediators expressed by HPMCs (obtained from donors of different ages) were determined in either basal conditions (non-stimulated) or after stimulation with HHb (10 mM) and normalized over its respective basal value. In (**a**) effects on NF-κB-dependent transcriptional activity are shown (assayed by transient transfection assay; $n=5$), and in (**b**) iNOS promoter activity ($n=4$); (**c**) RT–MPCR assay for iNOS/GAPDH mRNA ratio ($n=4$); (**d**) on NO_x levels (chemiluminescence method; $n=4$). Results are expressed as either fold induction of its basal for HHb-treated cells or RLUs (**a, b**), arbitrary units (**c**) and μM (**d**) for basal (non-stimulated) conditions.

markers, which was paralleled by an increase in basal levels of these markers, according to the previous results in our laboratory showing that age is accompanied by the presence of a basal inflammatory state in HPMC (Nevado *et al*., 2006).

Discussion and conclusions

Amadori adducts or early non-enzymatic glycated products have been shown to induce functional and structural changes in different cell types, including HPMCs (Peiró *et al*., 1998, 2003; Vallejo *et al*., 2000; Rodríguez-Mañas *et al*., 2003; Nevado *et al*., 2005). Furthermore, through the generation of ROS, Amadori adducts are able to induce proinflammatory responses (Nevado *et al*., 2005). The most outstanding finding of the present study is that the effect of Amadori adducts as inducers of a low-grade inflammatory response in HPMCs is impaired by the age of the donors of the HPMCs.

The most frequently studied non-enzymatic glycated products are the so-called AGEs (Cerami *et al*., 1998, Park *et al*., 2000), which exhibit proinflammatory properties (Luth *et al*., 2005) and have been detected in non-diabetic old people at higher levels than in younger ones (Wu *et al*., 2002; De Groot *et al*., 2004). As the presence of AGEs in our Amadori preparations was reasonably excluded, the results presented here seem to support that Amadori adducts may trigger a low-grade proinflammatory response in HPMCs in a way that is independent of the effects of AGEs.

It is worth noting that early glycated proteins can be incorporated into mesothelial cells by a transcytosis mechanism (Bodega *et al*., 2002). Indeed, the existence of non-enzymatic protein glycation has been also reported during continuous ambulatory peritoneal dialysis (CAPD) (Friedlander *et al*., 1996) in the mesothelial layer of the human peritoneum (Posthuma *et al*., 2001).

The proinflammatory properties of either HHb or gBSA, at concentrations that are close to free, physiological circulating levels (Tietz, 1990), in HPMC cultures, have been highlighted here in relevant systems involved in the inflammatory response, including the enzymes iNOS and COX-2, as well as several proinflammatory cytokines, all of them regulated by NF-κB (Go *et al*., 2005, Nevado *et al*., 2005), probably at a transcriptional level (Nevado *et al*., 2005). Interestingly, the effects of Amadori adducts on NF-κB-related proinflammatory markers were not observed or were very subtle, in HPMCs obtained from donors with ages between 65 and 81 years, where the basal or non-stimulated levels of those inflammatory markers were significantly increased, as we described previously (Nevado *et al*., 2006).

As is inferred from data obtained with HHb, a negative correlation between basal levels of several proinflammatory markers and Amadori-induced effects on the same pro-inflammatory substances was found over age, with a steeper decline at earlier ages and a smoother one after middle age. Therefore, it appears that the response induced by HHb is dependent upon the proinflammatory state, that is, the lower the basal response, the higher the stimulated (HHb-induced) one and vice versa. However, these age-associated effects elicited by HHb are not nonspecific, as in our same experimental HPMC model, the proinflammatory effect of

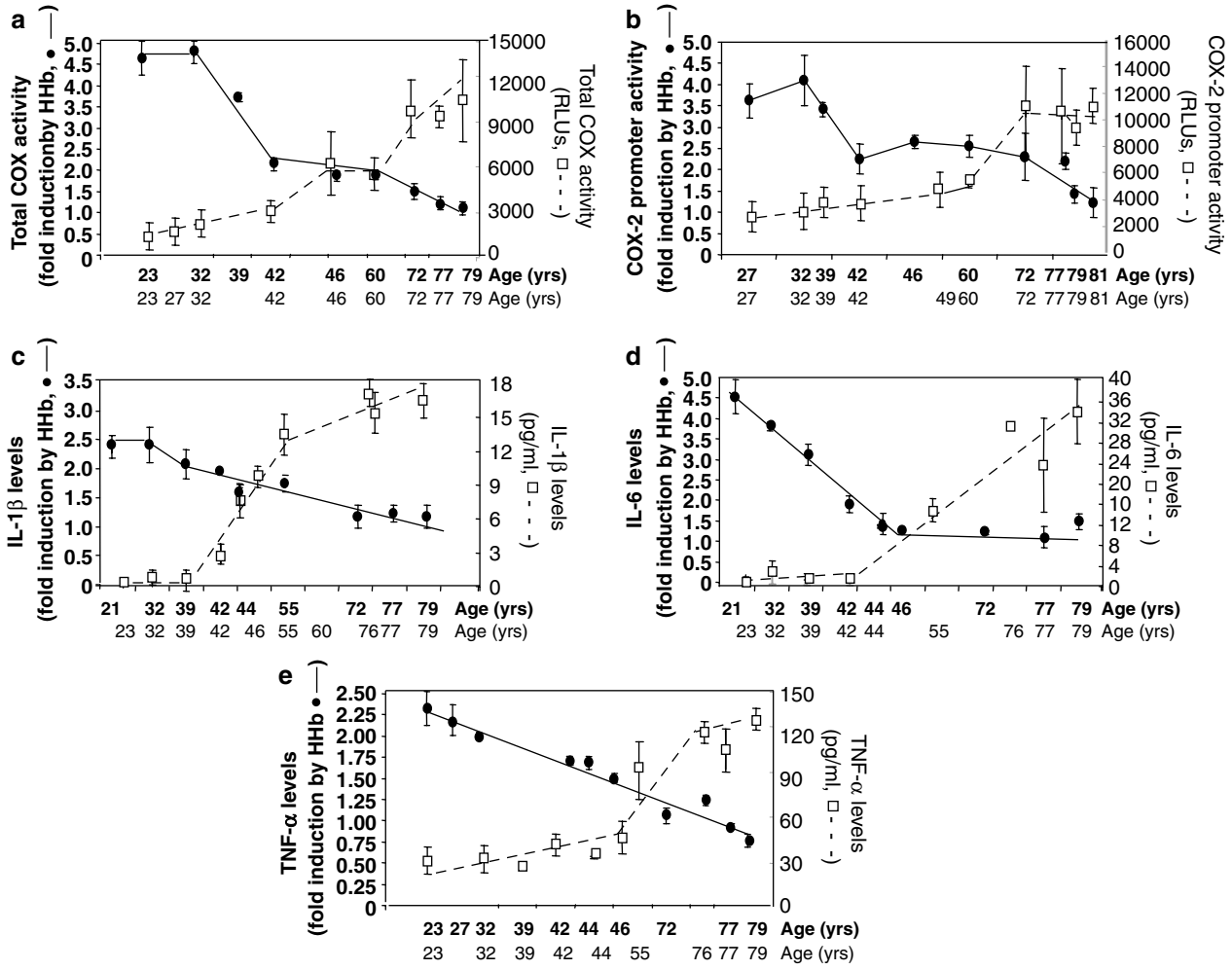

Figure 5 Correlations between basal levels of several proinflammatory markers and HHB-induced effects on same proinflammatory markers over age (part II). Activities of several proinflammatory mediators expressed by HPMCs (obtained from donors of different ages) were determined in either basal conditions (non-stimulated) or after stimulation with HHb (10 mM) and normalized over its respective basal value. In (**a**) effects on total COX activity are shown (luminescence assay; $n=4$); (**b**) on COX-2 ($-327/+59$) promoter activity transient transfection assay, $n=5$; (**c–e**) An ELISA-based assay for IL-1β, IL-6, and TNF-α levels, respectively ($n=4$). Results are expressed as either fold induction of its basal for HHb-treated cells or RLUs (**a, b**), and pg ml^{-1} (**c–e**) for basal (non-stimulated) conditions.

a mixture of cytokines was unaffected by the age of the donor. Furthermore, such age dependency is not observed for other regulatory systems in other cellular models of ageing. Thus, the effect of insulin or insulin-like growth factor-1 (IGF-1) on the proliferation and migration capacity of human smooth muscle cells is unaltered by age (Ruiz-Torres *et al*., 2003).

Finally, our data suggest that, HPMCs from young people seem to be more susceptible to the effects of early non-enzymatic glycated products as inducers of a low-grade proinflammatory response, than HPMC from older subjects, which are protected from this complication. However, findings in clinical practice would suggest the opposite. The clinical burden induced by diabetes in the elderly population is already high, not only in terms of complications in CAPD (higher rate of filtration failure and infectious complications), but also with an enhanced risk of major vascular complications. Therefore, factors others than Amadori adducts must be playing a role in the complications of diabetes with advancing age and it is tempting to suggest that the physiological ageing process may be able to modulate different pathways of cellular damage in diabetes.

In conclusion, our data suggest that the proinflammatory effects induced by Amadori adducts in HPMCs are strongly dependent on the age of the donor of the cells. Furthermore, the age dependence of cellular damage by glucose-related mechanisms explored here, raises the possibility that the mechanisms underlying the involvement of target organs in diabetes will be different, depending upon the clinical features of the disease, including the age of the patient. This possibility opens new opportunities for improving our knowledge about diabetes and its complications in elderly people, by exploring the biochemical and signalling mechanisms by which hyperglycaemia-linked events contribute to such complications in the ageing population. A more accurate knowledge will contribute to develop and implement more specific that is, age-targeted, therapeutic strategies, leading to a more successful treatment and prevention of the devastating consequences of diabetes.

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Conflict of interest

The authors state no conflict of interest.

References

Amore A, Cirina P, Mitola S, Peruzzi L, Gianoglio B, Rabbone I *et al*. (1997). Nonenzymatically glycated albumin (Amadori adducts) enhances nitric oxide synthase activity and gene expression in endothelial cells. *Kidney Int* **51**: 27–35.

Angulo J, Sánchez-Ferrer CF, Peiró C, Marín J, Rodríguez-Mañas L (1996). Impairment of endothelium-dependent relaxations by increasing percentages of glycated human haemoglobin. *Hypertension* **28**: 583–592.

Bodega F, Zacchi L, Agostini E (2002). Albumin transcytosis in mesothelium. *Am J Physiol Lung Cell Mol Physiol* **282**: L3–L11.

Cerami A, Vlassara H, Brownlee M (1998). Role of advanced glycosylation products in complications of diabetes. *Diabetes Care* **1**: 73–79.

Chung-Welch N, Patton WF, Shepro D, Cambria RP (1997). Two-stage isolation procedure for obtaining homogenous populations of microvascular endothelial and mesothelial cells from human omentum. *Microvascular Res* **54**: 121–134.

De Groot J, Verzijl N, Wenting-van Wijk MJ, Jacobs KM, Van Roermund PM, Bank RA *et al*. (2004). Accumulation of AGEs as a molecular mechanism of aging as a risk factor in osteoarthritis. *Arthritis Rheum* **50**: 1207–1215.

Ferrucci L, Ble A, Bandinelli S, Lauretani F, Suthers K, Guralnik JM (2004). A flame burning within. *Aging Clin Exp Res* **16**: 240–243.

Friedlander MA, Wu YC, Elgawish A, Monnier VM (1996). Early and advanced glycosylation end-products. Kinetics of formation and clearance in peritoneal dialysis. *J Clin Invest* **97**: 728–735.

Fries DM, Paxinou E, Themistocleous M, Swanberg E, Griendling KK, Salvemini D *et al*. (2003). Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells. *J Biol Chem* **25**: 22901–22907.

Go EK, Jun KJ, Kim JY, Yu BP, Chung HY (2005). Betaine supresses proinflammatory signaling during aging: the involvement of nuclear factor-κB via nuclear factor-inducing kinase/IκB kinase and mitogen-activated protein kinases. *J Gerontol: Biol Sci* **60A**: 1252–1264.

Hattori Y, Suziki M, Hattori S, Kasai K (2002). Vascular smooth muscle cell activation by glycated albumin (Amadori adducts). *Hypertension* **39**: 22–28.

Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T (1995). Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J Biol Chem* **270**: 24965–24971.

Luth HJ, Ogunlade V, Kuhla B, Kientsch-Engel R, Stahl P, Webster J *et al*. (2005). Age- and stage-dependent accumulation of advanced glycation end products in intracellular deposits in normal and Alzheimer's disease brains. *Cereb Cortex* **15**: 211–220.

Maggi S, Noale M, Gallina P, Marzari C, Bianchi D, Limongi F *et al*., ILSA Group (2004). Physical disability among older Italians with diabetes The ILSA study. *Diabetologia* **47**: 1957–1962.

Mandl-Weber S, Haslinger B, Schalkwijk CG, Sitter T (2001). Early glycated albumin, but not advanced glycated albumin, methylglyoxal, or 3-deoxyglucosonee increases the expression of PAI-1 in human peritoneal mesothelial cells. *Perit Dial Int* **21**: 487–494.

Nevado J, Peiró C, Vallejo S, El-Assar M, Lafuente N, Matesanz N *et al*. (2005). Amadori adducts activate NF-κB-related pro-inflammatory genes in human peritoneal mesothelial cells. *Br J Pharmacol* **146**: 268–279.

Nevado J, Vallejo S, El-Assar M, Peiró C, Sánchez-Ferrer CF, Rodríguez-Mañas L (2006). Changes in the human peritoneal mesothelial cells during aging. *Kidney Int* **69**: 313–322.

Ney KA, Colley KJ, Pizzo SV (1981). The standardization of the thiobarbituric acid assay for nonenzymatic glucosylation of human serum albumin. *Anal Biochem* **118**: 294–300.

Park MS, Lee HA, Chu WS, Yang DH, Hwang SD (2000). Peritoneal accumulation of AGE and peritoneal membrane permeability. *Perit Dial Int* **20**: 452–460.

Peiró C, Angulo J, Rodríguez-Mañas L, Llergo JL, Vallejo S, Cercas E *et al*. (1998). Vascular smooth muscle cell hypertrophy induced by glycosylated human oxyhaemoglobin. *Br J Pharmacol* **125**: 637–644.

Peiró C, Matesanz N, Nevado J, Lafuente N, Cercas E, Azcutia V *et al*. (2003). Glycated human oxyhaemoglobin activates nuclear factor-κB and activator protein-1 in cultured human aortic smooth muscle. *Br J Pharmacol* **140**: 681–690.

Posthuma N, ter Wee PM, Niessen H, Donker AJ, Vernrugh HA, Schalkwijk CG (2001). Amadori albumin and advanced glycation end-product formation in peritoneal dialysis using icodextrin. *Perit Dial Int* **21**: 43–51.

Rodríguez-Mañas L, Angulo J, Vallejo S, Peiró C, Sánchez-Ferrer A, Cercas E *et al*. (2003). Early and intermediate amadori glycated adducts oxidative stress, and endothelial dysfunction in the streptozotocin-induced diabetic rat's vasculature. *Diabetologia* **46**: 556–566.

Ruiz-Torres A, Lozano R, Melon J, Carraro R (2003). Age-dependent decline of *in vitro* migration (basal and stimulated by IGF-1 or insulin) of human vascular smooth muscle cells. *J Gerontol: Biol Sci* **58A**: 1074–1077.

Sarkar D, Lebedeva IV, Emdad L, Kang DC, Baldwin Jr AS, Fisher PB (2004). Human polynucleotide phosphorylase (hPNPaseold-35): a potential link between aging and inflammation. *Cancer Res* **64**: 7473–7478.

Sell DR, Monnier VM (1989). Structure elucidation of a senescence cross-link from human extracellular matrix. Implications of pentoses in the aging process. *J Biol Chem* **264**: 21597–21602.

Taylor BS, de Vera ME, Ganster RW, Wang K, Shapiro RA, Morris Jr SM *et al*. (1998). Multiple NF-κB enhancer elements regulate cytokine induction of human inducible nitric oxide synthase gene. *J Biol Chem* **273**: 15148–15156.

Tietz NW (1990). *Clinical Guide to Laboratory Tests*. WB Saunders Co.: Philadelphia, PA, pp 284–285.

Vallejo S, Angulo J, Peiró C, Nevado J, Sánchez-Ferrer A, Petidier R *et al*. (2000). Highly glycated oxyhemoglobin impairs nitric oxide relaxations in human mesenteric microvessels. *Diabetologia* **43**: 83–90.

Wu CH, Huang CM, Lin CH, Ho YS, Chen CM, Lee HM (2002). Advanced glycosylation end products induce NF-kappaB dependent iNOS expression in RAW 264.7 cells. *Mol Cell Endocrinol* **194**: 9–17.

Yañez-Mo M, Lara-Pezzi E, Selgas R, Ramírez-Huesca M, Domínguez-Jiménez C, Jiménez-Heffernan A *et al* (2003). Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. *N Engl J Med* **348**: 403–412.

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