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Vascular smooth muscle cell hypertrophy induced by glycosylated human oxyhaemoglobin

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1 Nonenzymatic protein glycosylation is a possible mechanism contributing to oxidative stress and vascular disease in diabetes. In this work, the influence of 14%-glycosylated human oxyhaemoglobin (GHHb), compared to the non-glycosylated protein (HHb), was studied on several growth parameters of rat cultured vascular smooth muscle cells (VSMC). A role for reactive oxygen species was also analysed.

2 Treatment of VSMC for 48 h with GHHb, but not with HHb, increased planar cell surface area in a concentration dependent manner. The threshold concentration was 10 nM, which increased cell size from 7965 ± 176 to 9411 ± 392 μm^2. Similarly, only GHHb enhanced protein content per well in VSMC cultures.

3 The planar surface area increase induced by 10 nM GHHb was abolished by superoxide dismutase (SOD; 50–200 u ml^{-1}), deferoxamine (100 nM–100 μM), or dimethylthiourea (1 mM), while catalase (50–200 u ml^{-1}) or mannitol (1 mM) resulted in a partial inhibition of cell size enhancement.

4 When a known source of oxygen free radicals was administered to VSMC, the xanthine/xanthine oxidase system, the results were analogous to those produced by GHHb. Indeed, enhancements of cell size were observed, which were inhibited by SOD, deferoxamine, or catalase.

5 These results indicate that, at low concentrations, GHHb induces hypertrophy in VSMC, this effect being mediated by superoxide anions, hydrogen peroxide, and/or hydroxyl radicals. Therefore, glycosylated proteins can have a role in the development of the structural vascular alterations associated to diabetes by enhancing oxidative stress.

Keywords: Glycosylated oxyhaemoglobin; superoxide anions; hydrogen peroxide; hydroxyl radicals; vascular smooth muscle; cell hypertrophy; diabetes

Introduction

Vascular diseases are a common consequence of long-term diabetes mellitus; indeed, microangiopathy, atherosclerosis and hypertension are more frequently observed in diabetic patients than in the general population (The National High Blood Pressure Education Program Working Group, 1994; Sowers & Epstein, 1995; Giugliano *et al*., 1996). Although the mechanisms involved in the development of diabetic vasculopathy are still not well understood, growing evidence has accumulated indicating that the generation of reactive oxygen species may play an important role in the etiology of these vascular complications (Tesfamariam, 1994; Pieper *et al*., 1995; Giugliano *et al*., 1996). Among the mechanisms that may contribute to increased oxidative stress in diabetes, nonenzymatic glycosylation of proteins has been proposed to play an important role (Baynes, 1991; Iino *et al*., 1996, Giugliano *et al*., 1996; Rumble *et al*., 1997).

Previous work from our laboratory (Rodríguez-Mañas *et al*., 1993; Angulo *et al*., 1996) provides evidence that human oxyhaemoglobin at nanomolar concentrations impairs endothelium-dependent relaxations when glycosylated at a percentage of 9% or higher. These nanomolar concentrations are in the range of the physiological circulating levels of free haemoglobin (Tietz, 1990). The inhibitory effect of glycosylated oxyhaemoglobin on endothelial responses is mediated by the release of reactive oxygen species, in particular superoxide anions, that inactivate endothelial nitric oxide (Angulo *et al*., 1996).

Recent reports indicate that reactive oxygen species can also stimulate vascular smooth muscle cell growth, by inducing the expression of growth-related proto-oncogenes (Rao & Berk, 1992; Rao *et al*., 1993a, 1996), or the activation of mitogen-activated protein kinases (Baas & Berk, 1995). Therefore, the aim of the present work was to analyse whether glycosylated human oxyhaemoglobin, as a source of reactive oxygen species, may influence vascular smooth muscle growth. For this purpose, we analysed different growth parameters of rat cultured vascular smooth muscle cells when exposed to non-glycosylated (HHb) or 14%-glycosylated human oxyhaemoglobin (GHHb). We also compared the effect of GHHb to that of a known source of reactive oxygen species, i.e., the xanthine/xanthine oxidase (XO) system or to another glycosylated protein, glycosylated human serum albumin (HSA).

Methods

Cell culture

Primary cultures of rat vascular smooth muscle cells (VSMC) were obtained by enzymatic dissociation of the aortae from normoglycemic 20-week old Sprague-Dawley rats (Iffa-Credo, Labresle, France) bred at the facilities of the Facultad de Medicina Autónoma (Madrid, Spain), as previously described (Peiró *et al*., 1997a). Briefly, the vessels were cleaned free of fat

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and connective tissue, cut into small pieces, placed in Dulbecco's Modified Eagle Medium (DMEM) containing 4 mg ml^{-1} collagenase (type II; Sigma Chemical Co. St Louis, Missouri, U.S.A.) and incubated for 90 min at 37°C in a humidified atmosphere of CO_2 (5%) and air (95%). The resulting cell suspension was then washed three times by centrifugation and seeded into 80-cm^2 culture flasks. Cells were characterized as vascular smooth muscle by two different criteria: (1) morphologically, confluent cultures exhibited a 'hill and valley' pattern typical of smooth muscle; (2) immunohistochemical staining with smooth muscle-specific monoclonal antibody to alpha-actin (Dakopatts, Glostrup, Denmark), using the avidin-biotin peroxidase complex method (Hsu *et al*., 1981), with more than 95% of the cells showing positive stain. VSMC were routinely grown in DMEM supplemented with 10% foetal calf serum, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2.5 μg ml^{-1} Amphotericin B. At confluence, cultures were passaged by trypsinization with 0.05% trypsin-0.02% EDTA. For experiments, cells between passages three and ten were used.

Determination of cell number and protein content per well

Cells were plated onto 24-well culture plates and grown in DMEM containing 10% foetal calf serum for 2–3 days. Medium was then switched to vehicle medium, i.e. serum-free DMEM containing 0.1% bovine serum albumin, either alone or containing the different compounds to be tested. VSMC were cultured for additional 48 h, with medium renewal after the first 24 h. Cell number per well was then determined based on a previously described procedure (Peiró *et al*., 1997b). Briefly, at the end of the treatment, cells were fixed with 1% glutaraldehyde, washed twice with phosphate-buffered saline, stained with 1% crystal violet (Fluka, Buchs, Switzerland) for 30 min and extensively washed with deionized water. After allowing plates to dry overnight, crystal violet was extracted from cells with 10% acetic acid and the resulting colour was measured by absorbance at 595 nm in an EL-340 automated microplate reader (Bio-Tek Instruments, Winooski, Vermont, U.S.A.). To validate this method for VSMC, a standard curve was carried out to establish the relationship between optical density and cell number counted by haemocytometer ($r=0.97$).

Protein content per well was determined in parallel plates. After solubilization of cultures with 0.2 N NaOH, protein was quantified by the method of Bradford (1976), using bovine serum albumin as standard.

Planar cell surface area

Planar cell surface area was determined according to a previously reported procedure (Peiró *et al*., 1997a). Briefly, VSMC were sparsely seeded onto 6-well culture plates in DMEM containing 10% foetal calf serum. After cell attachment, culture medium was switched to vehicle medium, either alone or containing the different compounds to be tested, and cultured for 48 h with medium renewal after the first 24 h. VSMC were then fixed with 1% glutaraldehyde and planar cell surface area was quantified by computer-assisted morphometry. Randomly selected images of VSMC were transmitted to a computer (Apple Macintosh Power 7100, Cupertino, California, U.S.A.) by a video camera (Sony Corporation, Tokyo, Japan) connected to the microscope (Nikon, Tokyo, Japan) and thereafter submitted to analysis with appropriate software (NIH Image). Measurements were performed in a blinded manner. In every experiment, at least 70 cells were counted for each treatment.

Materials

Culture plasticware was obtained from Costar (Cambridge, Massachussets, U.S.A.). DMEM, foetal calf serum and trypsin-EDTA were from Biological Industries (Beit Haemek, Israel). Oxyhaemoglobins were prepared by reduction of commercial non-glycosylated human haemoglobin and glycohaemoglobin A_1 (14% glycosylation; Sigma Chemical Co., St. Louis, Missouri, U.S.A.), as previously described (Angulo *et al*., 1996). A 50 mg ml^{-1} solution of human serum albumin (Sigma Chemical Co.) was glycosylated by incubation in phosphate-buffered saline (pH 7.4) containing 1 M glucose under sterilization for 6 days at 37°C, as previously described by others (Sakurai & Tsuchiya, 1988). Glycosylation of serum albumin was verified using the thiobarbituric acid assay (Ney *et al*., 1981). Superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes, catalase (EC 1.11.1.6) from bovine liver, xanthine oxidase (EC 1.1.3.22) from buttermilk and, unless otherwise stated, all other drugs or reagents were purchased from Sigma Chemical Co.

Statistical analysis

Values are given as means ± s.e.mean. The statistical analysis was evaluated by unpaired Student's *t*-test for single data points or by analysis of variance (ANOVA) for curves, with the level of significance chosen at $P<0.05$.

Results

Cell proliferation

After a 2-day treatment of confluent VSMC with HHb or GHHb (1 nM – 1 μM), basal cell number per well was not significantly affected, except with the highest concentration used (Figure 1a). Indeed, both HHb and GHHb at a concentration of 1 μM promoted cell proliferation to a similar extent ($P<0.05$ by unpaired Student's *t*-test). When cells were treated in similar conditions with 100 μM xanthine/XO (1 – 100 μu ml^{-1}), no significant changes in basal cell number per well were observed (Figure 1b). Stimulation with 10% foetal calf serum, used as a positive control, yielded a 3.20 ± 0.16 fold increase in cell number per well (results from three independent experiments, $P<0.05$ by unpaired Student's *t*-test).

Effect of HHb and GHHb on planar cell surface area

Planar cell surface area in basal conditions, i.e. when VSMC were maintained for 2 days in vehicle medium, was 7965 ± 176 μm^2 (results obtained from 20 independent experiments, counting at least 70 cells per experiment). After treatment of VSMC with GHHb (1 nM to 1 μM) for the same time period, a concentration-dependent increase in planar cell surface area was observed (Figure 2). The threshold concentration for GHHb was of 10 nM, which enhanced cell size to 9411 ± 392 μm^2, while 1 μM yielded a planar cell surface area value of 11,413 ± 454 μm^2. On the other hand, HHb did not modify cell size at nanomolar concentrations (Figure 2). Only at a concentration of 1 μM did HHb produce a significant enhancement of planar cell surface area to 8865 ± 350 μm^2.

In addition, treatment of VSMC with 10 nM GHHb also resulted in enhanced protein content per well while 10 nM

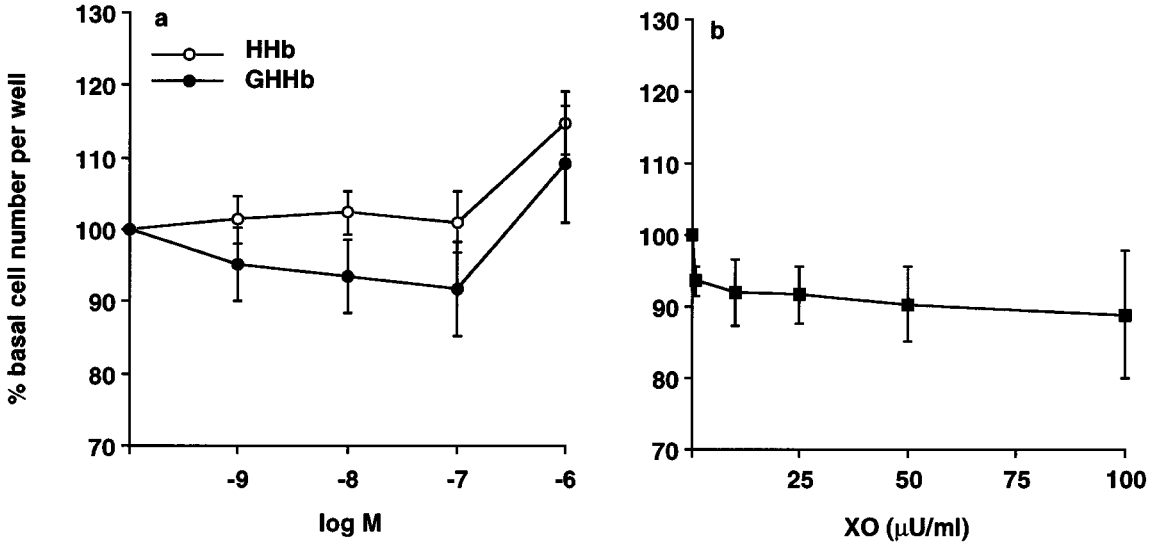

Figure 1 Effect of (a) non-glycosylated human oxyhaemoglobin (HHb) and 14%-glycosylated human oxyhaemoglobin (GHHb), and (b) xanthine (100 μM)/ xanthine oxidase (XO) on cell proliferation. Results (mean $\pm$ s.e.mean) are expressed as percentage of cell number achieved in basal conditions, which averaged 322,031 $\pm$ 21,127 cells per well. Data were obtained from at least five independent experiments.

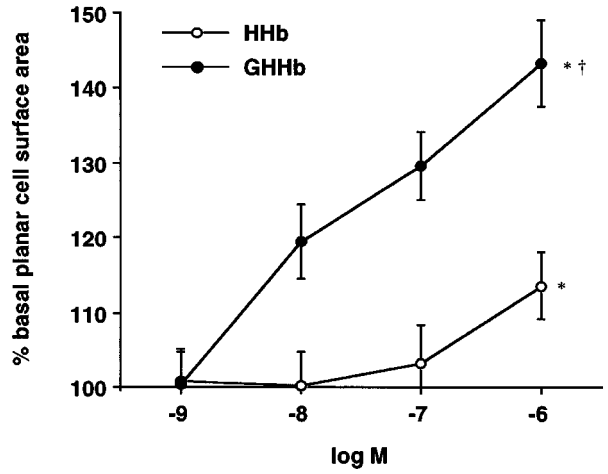

Figure 2 Effect of non-glycosylated human oxyhaemoglobin (HHb) and 14%-glycosylated human oxyhaemoglobin (GHHb) on cell size. Results (mean $\pm$ s.e.mean) are expressed as percentage of planar cell surface area observed in basal conditions, which averaged 7965 $\pm$ 176 μm^2. Data were obtained from five independent experiments. *$P<0.05$ *versus* basal cell size (one-way ANOVA); †$P<0.05$ between HHb and GHHb (two-way ANOVA).

HHb did not significantly modify this parameter (from 54.2 $\pm$ 1.9 μg well^{-1} in basal conditions to 60.6 $\pm$ 0.8 and 56.5 $\pm$ 0.7 μg well^{-1} in the presence of GHHb and HHb, respectively; results from three independent measurements, $P<0.05$ for GHHb by unpaired Student's *t*-test).

To study the possible involvement of reactive oxygen species on the increase in cell size induced by 10 nM GHHb, VSMC cultures were treated with different scavengers of oxygen species. Figure 3a shows that the increase in planar cell surface area induced by 10 nM GHHb was completely abolished by increasing concentrations of SOD (50–200 u ml^{-1}), while treatment with catalase (50–200 u ml^{-1}) resulted in a partial decrease of the cell size increase, which was reduced by 55%. Co-incubation with 200 u ml^{-1} catalase did not modify the inhibitory action of 200 u ml^{-1} SOD on the enhancement of cell size elicited by 10 nM GHHb (99.2 $\pm$ 3.3% and 102.5 $\pm$ 4.4% of basal planar cell surface area, in the presence of SOD or in the presence of SOD plus catalase, respectively; results from three independent experiments).

Deferoxamine (100 nM – 100 μM) also reversed the effect of 10 nM GHHb on cell size in a concentration-dependent manner (Figure 3b). Additionally, as shown in Figure 4, the effect of 10 nM GHHb was reduced to 45% by mannitol (1 mM) and completely abolished by dimethylthiourea (DMTU; 1 mM).

SOD (200 u ml^{-1}), catalase (200 u ml^{-1}), deferoxamine (100 μM), mannitol (1 mM) or DMTU (1 mM) failed to modify cell size by themselves (99.1 $\pm$ 1.9%, 101.3 $\pm$ 1.6%, 98.1 $\pm$ 1.1%, 102.29 $\pm$ 1.31% and 98.89 $\pm$ 1.69% of basal planar cell surface area, respectively; results from three independent experiments).

Effect of glycosylated HSA on VSMC size

To assess whether other nonenzymatically glycosylated proteins could have an effect similar to that of GHHb on VSMC size, cultures were treated with glycosylated HSA. Glycosylated HSA (0.5–5 mg ml^{-1}) induced a concentration-dependent enhancement of planar cell surface area, as shown in Figure 5. Non-glycosylated HSA used at the same concentration range produced no significant changes in planar cell surface area (Figure 5).

The effect of 2 ng ml^{-1} glycosylated HSA on cell size was abolished by either SOD (200 u ml^{-1}) or deferoxamine (100 μM) (96.2 $\pm$ 2.9% and 99.9 $\pm$ 1.2% of basal planar cell surface area, respectively; results from three independent experiments, $P<0.05$ *versus* 2 ng ml^{-1} glycosylated HSA by unpaired Student's *t*-test).

Effect of xanthine/XO on planar cell surface area

The effect of XO on VSMC size is shown in Figure 6. Incubation of xanthine (100 μM) with increasing concentrations of XO (1–300 μu ml^{-1}) resulted in a concentration-

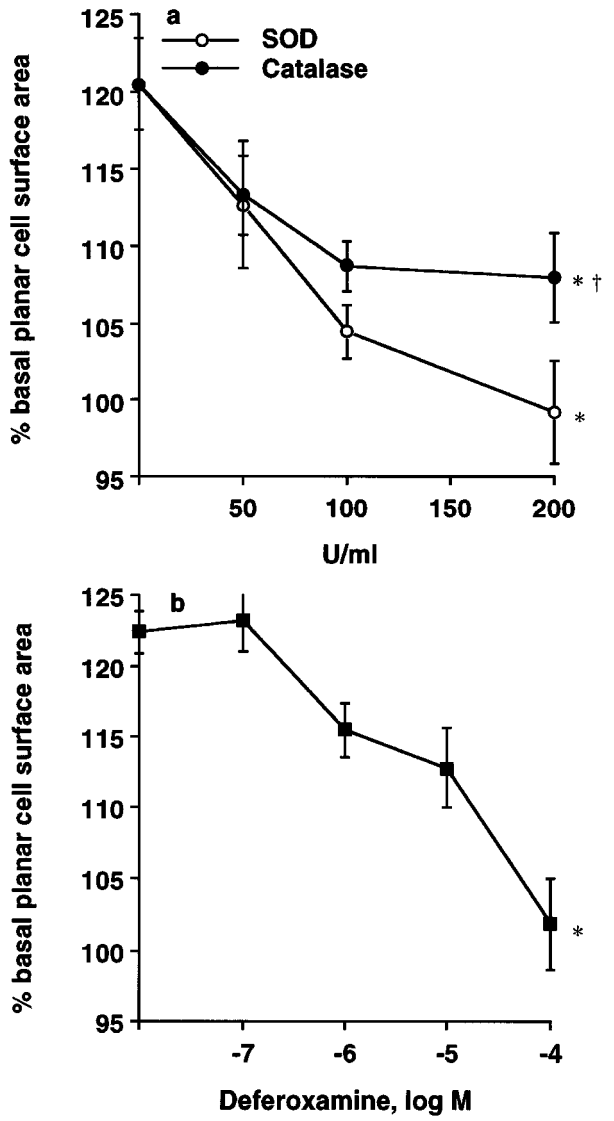

Figure 3 Effect of (a) superoxide dismutase (SOD) or catalase, and (b) deferoxamine on the increase in cell size elicited by 14%-glycosylated human oxyhaemoglobin (10 nM). Results (mean ± s.e.mean) are expressed as percentage of planar cell surface area observed in basal conditions. Data were obtained from five independent experiments. $*P<0.05$ *versus* GHHb-induced cell size increase (one-way ANOVA); $\dagger P<0.05$ between SOD and catalase (two-way ANOVA).

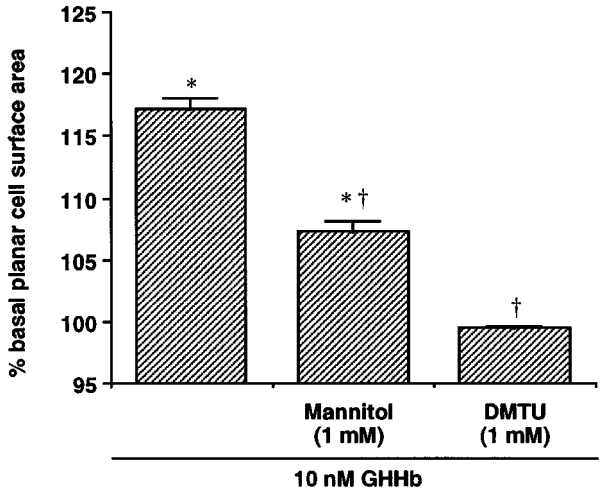

Figure 4 Effect of mannitol and dimethylthiourea (DMTU) on the increase in cell size elicited by 14%-glycosylated human oxyhaemoglobin (GHHb). Results (mean ± s.e.mean) are expressed as percentage of planar cell surface area observed in basal conditions. Data were obtained from three independent experiments. $*P<0.05$ *versus* basal; $\dagger P<0.05$ *versus* GHHb-induced cell size increase.

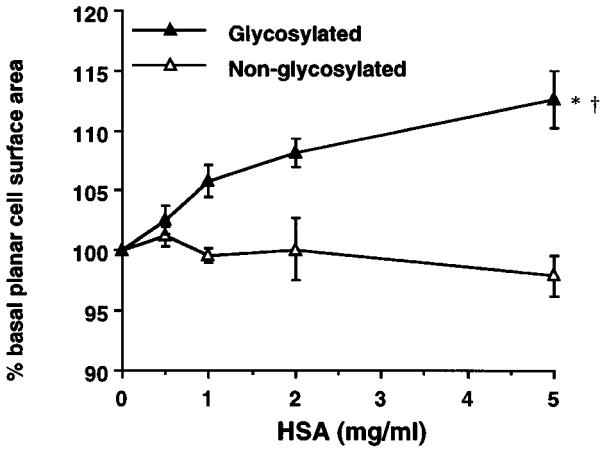

Figure 5 Effect of glycosylated and non-glycosylated human serum albumin (HSA) on cell size. Results (mean ± s.e.mean) are expressed as percentage of planar cell surface area observed in basal conditions. Data were obtained from three independent experiments. $*P<0.05$ *versus* basal cell size (one-way ANOVA); $\dagger P<0.05$ between glycosylated and non-glycosylated HSA (two-way ANOVA).

dependent increase of planar cell surface area. Threshold concentration of XO was 5 μu ml^{-1} and maximal effect was observed at 100 μu ml^{-1}XO, enhancing planar cell surface area values from 7965 ± 176 μm^2 to 9289 ± 360 μm^2 and $11{,}031 \pm 618$ μm^2, respectively (results from five independent experiments). When used at concentrations higher than 1 mu ml^{-1} XO produced marked cell death, as assessed by light microscopy. Xanthine alone did not modify basal VSMC size (102.6 ± 3.2% of basal planar cell surface area; results from three independent experiments).

The increase in cell size induced by xanthine/XO was accompanied by enhanced protein content per well from 54.2 ± 1.9 μg well^{-1} in basal conditions to 60.1 ± 1.1 μg well^{-1} in the presence of 10 μu ml^{-1} XO, which yields an increase in basal cell size similar to that obtained with 10 nM GHHb (results from three independent measurements, $P<0.05$ by unpaired Student's *t*-test).

The effect of a concentration of 10 μu ml^{-1} XO was completely abolished by the XO inhibitor allopurinol (100 μM) (100.8 ± 4.4% of basal cell size, results from three independent experiments). Allopurinol alone failed to modify basal planar cell surface area at the concentration used in the present study (96.4 ± 4.2% of basal size, results from three independent experiments). Moreover, treatment with both SOD or catalase (25–200 u ml^{-1}) abolished the effect of 10 μu ml^{-1} XO in a concentration-dependent manner, as shown in Figure 7a. A similar inhibitory effect on XO-induced increase in cell size was obtained after treatment with increasing concentrations of deferoxamine (100 nM – 10 μM) (Figure 7b).

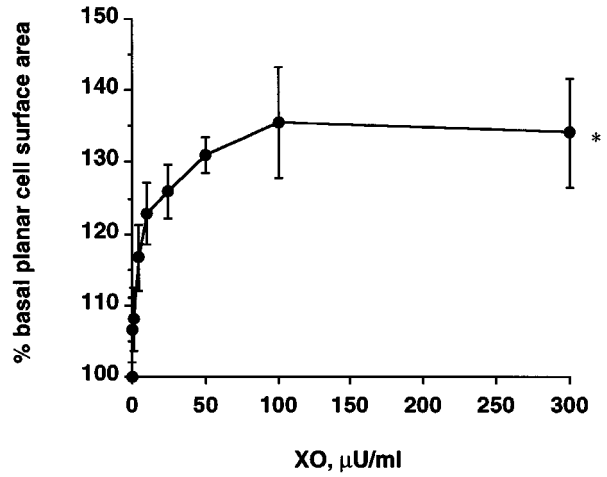

Figure 6 Effect of xanthine (100 μM)/ xanthine oxidase (XO) on cell size. Results (mean ± s.e.mean) are expressed as percentage of planar cell surface area observed in basal conditions. Data were obtained from five independent experiments. *$P<0.05$ *versus* basal cell size (one-way ANOVA).

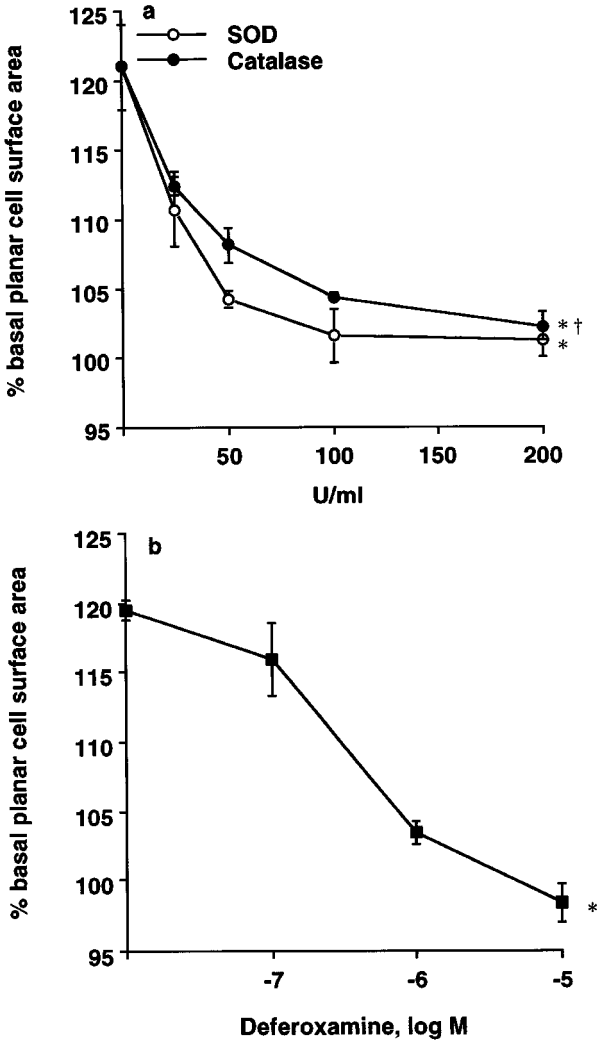

Figure 7 (a) Effect of superoxide dismutase (SOD) or catalase, and (b) deferoxamine on the increase in cell size elicited by 10 μu ml^{-1} XO. Results (mean ± s.e.mean) are expressed as percentage of planar cell surface area observed in basal conditions. Data were obtained from five independent experiments. *$P<0.05$ *versus* XO-induced cell size increase (one-way ANOVA); †$P<0.05$ between SOD and catalase (two-way ANOVA).

Discussion

Vascular hypertrophy has been implicated in the pathogenesis of diabetic vasculopathy, leading to associated hypertension, nephropathy, and atherosclerosis (Williamson *et al*., 1988; Sowers & Epstein, 1995). Enhanced thickness of arterial medial layer together with extracellular matrix expansion has been observed in experimental models of diabetes (Cooper *et al*., 1994; Öztürk *et al*., 1996; Rumble *et al*., 1996, 1997). In addition, several *in vitro* studies have shown enhanced VSMC growth associated with diabetes; indeed, it has been reported that VSMC isolated from diabetic patients or from animal models of diabetes exhibit higher proliferation rates than their respective non-diabetic controls (Alipui *et al*., 1993; Oikawa *et al*., 1996; Parès-Herbuté *et al*., 1996). Furthermore, increased proliferation rates have also been reported in VSMC cultured in a high glucose environment (Graier *et al*., 1995).

Different mechanisms have been proposed to be involved in the structural vascular alterations observed in long-term diabetes, including the renin-angiotensin system (Cooper *et al*., 1994), the kinin-nitric oxide pathway (Rumble *et al*., 1996), several growth factors (Rumble *et al*., 1997), or some glucose-dependent mechanisms, including nonenzymatic protein glycation and advanced glycosylation end product formation (Iino *et al*., 1996; Rumble *et al*., 1997). In addition, there is growing evidence indicating that increased oxidative stress in blood vessels is a crucial event in the etiology of diabetic vascular complications (Tesfamariam, 1994; Pieper *et al*., 1995; Giugliano *et al*., 1996). Enhanced reactive oxygen species production may be the consequence of increased pro-oxidant factors, decreased endogenous antioxidant defenses, or both (Giugliano *et al*., 1996). It has been proposed that the activity of natural detoxifying enzymes, such as SOD, catalase or glutathione peroxidase may be decreased in diabetes, although there are marked discrepancies between the different studies reported (Pieper *et al*., 1995; Giugliano *et al*., 1996). Concerning the enhancement of reactive oxygen species production in diabetes, hyperglycemia is a key factor that leads to increased oxidative stress by several mechanisms, such as glucose autoxidation or nonenzymatic glycosylation of proteins (Hunt *et al*., 1988; Vlassara *et al*., 1994).

In previous work, we have analysed the role of glycosylated human oxyhaemoglobin on vascular reactivity (Rodríguez-Mañas *et al*., 1993; Angulo *et al*., 1996). Our interest in oxyhaemoglobin resides in: (1) its degree of glycosylation is very sensitive to changes in circulating glucose levels (Nathan *et al*., 1984); (2) it can be found free in plasma at low concentrations (Tietz, 1990); and (3) it can penetrate into the vascular wall, therefore reaching different vascular cell types (Stary *et al*., 1992). We have reported evidence indicating that glycosylated human oxyhaemoglobin inactivates endothelial nitric oxide by releasing superoxide anions (Angulo *et al*., 1996). In the present study, we aimed to analyse whether human glycosylated oxyhaemoglobin, through the release of reactive oxygen species, may modulate not only vascular function but also vascular structure by promoting VSMC growth. For this purpose, we tested whether GHHb could affect some growth parameters of cultured VSMC, such as cell proliferation or cell size, and we compared its effects to those of HHb.

Treatment of VSMC cultures with increasing concentrations of either HHb or GHHb did not affect cell proliferation except at high micromolar concentrations, where both compounds elicited a similar increase in cell number per well. However, despite the lack of effect on cell proliferation, GHHb induced a concentration-dependent increase in planar cell surface area, which was already significant at a concentration

of 10 nM. GHHb also resulted in increased protein content of VSMC cultures. This effect was not observed with HHb, which only at micromolar concentrations produced a modest increase in cell size. The effect of GHHb on cell size and protein content, without affecting cell proliferation, indicates that at nanomolar concentrations GHHb is acting as a trophic factor for VSMC by promoting cell hypertrophy.

As we have previously indicated that glycosylated human oxyhaemoglobin may release superoxide anions (Angulo *et al*., 1996), we aimed to determine the possible role for this and other reactive oxygen species in mediating the effect of GHHb on VSMC growth. Indeed, in the presence of SOD (scavenger of superoxide anions), the enhancement of planar cell surface area induced by a 10 nM concentration of GHHb was completely inhibited in a concentration-dependent manner. This effect of SOD was not thought likely to be due to an enhanced production of hydrogen peroxide (Mullarkey *et al*., 1990) because it was not affected by catalase. In fact, catalase by itself (a scavenger of hydrogen peroxide) also reduced the hypertrophic response to GHHb to almost a half. Similarly, deferoxamine (which prevents the formation of hydroxyl radicals) abolished the cell size increase evoked by GHHb in a concentration-dependent manner. These results indicate that either superoxide anions or hydroxyl radicals, as well as hydrogen peroxide to a lesser degree, may be involved in the hypertrophic effect of GHHb.

Superoxide anions are most likely released by GHHb in the extracellular space (as SOD does not enter the cell). Supporting this, several reports indicate the ability of different nonenzymatically glycosylated proteins, such as serum albumin or ribonuclease, to release reactive oxygen species, especially superoxide anions (Sakurai & Tsuchiya, 1988; Mullarkey *et al*., 1990). Although HHb may also release superoxide anions due to autoxidation (Misra & Fridovich, 1972; Wever *et al*., 1973), we suggest that, at a given concentration, the amount of superoxide anions released is significantly higher for GHHb than for HHb, so that the superoxide anions released by GHHb at nanomolar concentrations may be still functionally relevant, while that produced by HHb would be negligible.

As previously indicated, in addition to superoxide anions, hydroxyl radicals and hydrogen peroxide may have a role in mediating the hypertrophic responses to glycosylated oxyhaemoglobin. This finding contrasts with the interference of endothelium-dependent relaxations by this agent, which is mainly mediated by superoxide anions with no apparent role for other reactive oxygen species (Angulo *et al*., 1996). However, it is in agreement with those reports indicating that different reactive oxygen species may modulate VSMC growth (Rao & Berk, 1992; Rao *et al*., 1993a; Baas & Berk, 1995). Thus, reactive oxygen species can affect several growth-related pathways in VSMC, such as the expression of c-*fos* or c-*jun* proto-oncogenes (Rao & Berk, 1992; Rao *et al*., 1993a) or the activation of mitogen-activated protein kinases (Rao *et al*., 1993b; Baas & Berk, 1995; Guyton *et al*., 1996). In addition, reactive oxygen species may also modulate the effect of some growth factors (Herbert *et al*., 1996).

At present, we cannot conclude whether hydroxyl radicals and hydrogen peroxide are directly released by GHHb or these reactive oxygen species derive from superoxide anions, as superoxide anions can be rapidly converted into hydrogen peroxide (Griendling & Ushio-Fukai, 1997). In addition, once released, superoxide anions may react with hydrogen peroxide to form, *via* the Haber-Weiss and Fenton reactions, hydroxyl radicals (Gutteridge *et al*., 1981; Kukreja & Hess, 1994). This generation of hydroxyl radicals requires reduced iron (Fe^{2+}) as a cofactor (Gutteridge *et al*., 1981; Cohen, 1994). It is interesting to note that, in our experimental conditions, Fe^{2+} is contained in the culture medium (DMEM). The results obtained with deferoxamine suggest that hydroxyl radicals are likely to be the active mediator of the hypertrophic effect of GHHb. To further confirm this hypothesis, experiments were performed in the presence of selective hydroxyl radical scavengers such as mannitol and DMTU. Although mannitol failed to completely abolish the effect of GHHb, this could be achieved in the presence of DMTU. These results indicate, (1) that hydroxyl radicals are generated outside the cell, *via* the aboved described reactions, as mannitol is an extracellular scavenger (Tan *et al*., 1995), and (2) hydroxyl radicals presumably cross the plasma membrane to act inside VSMC, as DMTU is an intracellular hydroxyl radicals scavenger (Tan *et al*., 1995).

In order to verify whether the ability of GHHb to promote VSMC hypertrophy was shared by other nonenzymatically glycosylated proteins, cultures were treated with glycosylated HSA. Indeed, glycosylated HSA, but not non-glycosylated HSA, promoted cell hypertrophy, this effect being mediated by superoxide anions and hydroxyl radicals, as it was abolished by both SOD and deferoxamine. Glycosylated HSA appears therefore to share hypertrophic mechanisms with GHHb, although GHHb seems to be more efficient in promoting VSMC hypertrophy. Indeed, to achieve a similar increase in VSMC size a much lower concentration of GHHb is needed (10 nM GHHb *versus* 5 mg ml^{-1} glycosylated HSA, which is grossly equivalent to 100 mM, to obtain a 15% increase in planar cell surface area).

We further aimed to validate the results obtained using a well established source of reactive oxygen species, the xanthine/XO system, known to activate growth-related mechanisms in VSMC (Rao & Berk, 1992; Baas & Berk, 1995). XO is a source of superoxide anions, hydrogen peroxide, and hydroxyl radicals, although superoxide is the main reactive oxygen species directly released by XO (Cohen, 1994). In the present experimental conditions, XO yielded similar results to those obtained with GHHb; XO did not affect cell proliferation but rather promoted increased cell size and protein content of VSMC. The hypertrophic effect produced by 10 μu ml^{-1} XO, which was quantitatively similar to that induced by 10 nM GHHb, was analogously inhibited by SOD, catalase, or deferoxamine, indicating the participation of the same reactive oxygen species. It is worth mentioning that XO seemed to be more sensitive to the scavengers of reactive oxygen species than GHHb. Although we have no clear explanation for this, it is recognized that the effects induced by XO *in vitro* are effectively quenched by rather small amounts of SOD or catalase (Beckman, 1994).

In conclusion, we propose that glycosylated oxyhaemoglobin, a marker of glycaemic control in diabetes, at concentrations that can be found free in plasma (Tietz, 1990), may act as a source of reactive oxygen species. Therefore, glycosylated oxyhaemoglobin, and probably other nonenzymatically glycosylated proteins, either in blood or into the vascular wall, can have a pathophysiological role in the development of the structural vascular alterations associated to diabetes through the enhancement of oxidative stress. To our knowledge, there are no previous studies analysing the role of these reactive oxygen species as direct promoters of VSMC hypertrophy and, therefore, the possible mechanisms involved remain to be elucidated. In our opinion, however, this may be a relevant event in the development of diabetic vascular disease.

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References

ALIPUI, C., RAMOS, K. & TENNER, T.E. Jr. (1993). Alterations of rabbit aortic smooth muscle cell proliferation in diabetes mellitus. *Cardiovasc. Res.,* **27,** 1229 – 1232.

ANGULO, J., SÁNCHEZ-FERRER, C.F., PEIRÓ, C., MARÍN, J. & RODRÍGUEZ-MAÑAS, L. (1996). Impairment of endothelium-dependent relaxation by increasing percentages of glycosylated human hemoglobin. Possible mechanisms involved. *Hypertension,* **28,** 583 – 592.

BAAS, A.S. & BERK, B.C. (1995). Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2^- in vascular smooth muscle cells. *Circ. Res.,* **77,** 29 – 36.

BAYNES, J.W. (1991). Role of oxidative stress in the development of complications in diabetes. *Diabetes,* **40,** 405 – 412.

BECKMAN, J.S. (1994). Peroxynitrite versus hydroxyl radical: the role of nitric oxide in superoxide-dependent cerebral injury. *Ann. N.Y. Acad. Sci.,* **738,** 69 – 75.

BRADFORD, M.M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 – 254.

COHEN, G. (1994). Enzymatic/Nonenzymatic sources of oxyradicals and regulation of antoxidant defenses. *Ann. N.Y. Acad. Sci.,* **738,** 8 – 14.

COOPER, M.E., RUMBLE, J.R., KOMERS, R., HE-CHENG, D, JANDELEIT, K. & SHEUNG-TO, C. (1994). Diabetes-associated mesenteric vascular hypertrophy is attenuated by angiotensin-converting enzyme inhibition. *Diabetes,* **43,** 1221 – 1228.

GIUGLIANO, D., CERIELLO, A. & PAOLISSO, G. (1996). Oxidative stress and diabetic vascular complications. *Diabetes Care,* **19,** 257 – 267.

GRAIER, W.F., GRUBENTHAL, I., DITTRICH, P., WASCHER, T.C. & KOSTNER, G.M. (1995). Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation. *Eur. J. Pharmacol.,* **294,** 221 – 229.

GRIENDLING, K.K. & USHIO-FUKAI, M. (1997). NADH/NADPH oxidase and vascular function. *Trends. Pharmacol. Sci.,* **7,** 301 – 307.

GUTTERIDGE, J.M., ROWLEY, D.A. & HALLIWELL, B. (1981). Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. *Biochem. J.,* **199,** 263 – 265.

GUYTON, K.Z., LIU, Y., GOROSPE, M., XU, K. & HOLBROOK, N. (1996). Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. *J. Biol. Chem.,* **271,** 4138 – 4142.

HERBERT, J.M., BONO, F. & SAVI, P. (1996). The mitogenic effect of H_2O_2 for vascular smooth muscle cells is mediated by an increase of the affinity of basic fibroblast growth factor for its receptor. *FEBS Lett.,* **395,** 43 – 47.

HSU, S.M., RAINE, L. & FANGER, H. (1981). Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase technique. A comparison between ABC and unlabelled antibody (PAP) procedures. *J. Hystochem. Cytochem.,* **29,** 577 – 580.

HUNT, J.V., DEAN, R.T. & WOLFF, S.P. (1988). Hydroxyl radical production and autoxidative glycosylation: glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochem. J.,* **256,** 205 – 212.

IINO, K., YOSHINARI, M., YAMAMOTO, M., KAKU, K., DOI, Y., ICHIKAWA, K., IWASE, M. & FUJISHIMA, M. (1996). Effect of glycated collagen on proliferation of human smoth muscle cells in vitro. *Diabetologia,* **39,** 800 – 806.

KUKREJA, R.C. & HESS, M.L. (1994). Biochemistry of oxygen and nitrogen metabolites. In *Free Radicals, Cardiovascular Dysfunction and Protection Strategies*. ed. Medical Intelligence Unit. pp. 1 – 8. Austin: RG Landes Co.

MISRA, H.P. & FRIDOVICH, I. (1972). The generation of superoxide radical during the autooxidation of hemoglobin. *J. Biol. Chem.,* **247,** 6960 – 6962.

MULLARKEY, C.J., EDELSTEIN, D. & BROWNLEE, M. (1990). Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem. Biophys. Res. Commun.,* **173,** 932 – 939.

NATHAN, D.M., SINGER, D.E., HURXTHAL, K. & GOODSON, J.D. (1984). The clinical information value of the glycosylated hemoglobin assay. *N. Engl. J. Med.,* **310,** 341 – 346.

NEY, K.A., COLLEY, K.J. & PIZZO, S.V. (1981). The standardization of the thiobarbituric acid assay for nonenzymatic glucosylation of human serum albumin. *Anal. Biochem.,* **118,** 294 – 300.

OIKAWA, S., HAYASAKA, K., HASHIZUME, E., KOTAKE, H., MIDORIKAWA, H., SEKIKAWA, A., KIKUCHI, A. & TOYOTA, T. (1996). Human arterial smooth muscle cell proliferation in diabetes. *Diabetes,* **45** (suppl. 3), S114 – S116.

ÖZTÜRK, Y., ALTAN, V.M. & YILDIZOGLU-ARIN, N. (1996). Effects of experimental diabetes and insulin on smooth muscle functions. *Pharmacol. Rev.,* **48,** 69 – 112.

PARÈS-HEBUTÉ, N., HILLAIRE-BUYS, P., GROSS, R., LOUBATIÈRES-MARIANI, M.M. & MONNIER, L. (1996). Adenosine inhibitory effect on enhanced growth of aortic smooth muscle cells from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.,* **118,** 783 – 789.

PEIRÓ, C., ANGULO, J., REGADERA, J., LLERGO, J.L., SÁNCHEZ-FERRER, A., RODRÍGUEZ-MAAS, L. & SÁNCHEZ-FERRER, C.F. (1997b). Nifedipine, losartan and captopril effects on hyperplasia of vascular smooth muscle from Ren-2 transgenic rats. *Eur. J. Pharmacol.,* **324,** 257 – 265.

PEIRÓ, C., LLERGO, J.L., ANGULO, J., LÓPEZ-NOVOA, J.M., RODRÍGUEZ-LÓPEZ, A., RODRÍGUEZ-MAÑAS, L. & SÁNCHEZ-FERRER, C.F. (1997a). Effects of captopril, losartan, and nifedipine on cell hypertrophy of cultured vascular smooth muscle from hypertensive Ren-2 transgenic rats. *Br. J. Pharmacol.,* **121,** 1438 – 1444.

PIEPER, G.M., JORDAN, M., DONDLINGER, L.A., ADAMS, M.B. & ROZA, A.M. (1995). Peroxidative stress in diabetic blood vessels. Reversal by pancreatic islet transplantation. *Diabetes,* **44,** 884 – 889.

RAO, G.N. & BERK, B.C. (1992). Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ. Res.,* **70,** 593 – 599.

RAO, G.N., GLASGOW, W.C., ELING, T.E. & RUNGE, M.E. (1996). Role of hydroperoxyeicosatetranoic acids in oxidative stress-induced activating protein 1 (AP-1) actvity. *J. Biol. Chem.,* **271,** 27760 – 27764.

RAO, G.N., LASSEGUE, B., GRIENDLING, K.K. & ALEXANDER, R.W. (1993a). Hydrogen peroxide stimulates transcription of c-jun in vascular smooth muscle cells: role of arachidonic acid. *Oncogene,* **8,** 2759 – 2764.

RAO, G.N., LASSÉGUE, B., GRIENDLING, K.K., ALEXANDER, R.W. & BERK, B.C. (1993b). Hydrogen peroxide-induced c-*fos* expression is mediated by arachidonic acid release: role of protein kinase C. *Nucl. Acid Res.,* **21,** 1259 – 1263.

RODRÍGUEZ-MAÑAS, L., ARRIBAS, S., GIRON, C., VILLAMOR, J., SÁNCHEZ-FERRER, C.F. & MARÍN, J. (1993). Interference of glycosylated human hemoglobin with endothelium-dependent responses. *Circulation,* **88,** 2111 – 2116.

RUMBLE, J.R., COOPER, M.E., SOULIS, T., COX, A., WU, L., YOUSSEF, S., JASIK, M. & JERUMS, G. (1997). Vascular hypertrophy in experimental diabetes. Role of advanced glycation end products. *J. Clin. Invest.,* **99,** 1016 – 1027.

RUMBLE, J.R., KOMERS, R. & COOPER, M.E. (1996). Kinins or nitric oxide, or both, are involved in the antitrophic effects of angiotensin converting enzyme inhibitors on diabetes-associated mesenteric vascular hypertrophy in the rat. *J. Hypertens.,* **4,** 601 – 607.

SAKURAI, T. & TSUCHIYA, S. (1988). Superoxide production from nonenzymatically glycated protein. *FEBS Lett.,* **236,** 406 – 410.

SHENG, M. & GREENBERG, M.E. (1990). Deregulation of an function of c-fos and other immediate early genes. *Neuron,* **4,** 477 – 485.

SOWERS, J.R. & EPSTEIN, M. (1995). Diabetes mellitus and associated hypertension, vascular disease, and nephropathy. An update. *Hypertension,* **26,** 869 – 879.

STARY, H.C., BLANKENHORN, D.H., CHANDLER, A.B., GLAGOV, S., INSULL, W. Jr, RICHARDSON, M., ROSENFELD, M.E., SCHAFFER, S.A., SCHWARTZ, C.J., WAGNER, W.D. & WISSLER, R.W. (1992). A definition of the intima of human arteries and of its atherosclerosis-prone regions. *Circulation,* **85,** 391 – 405.

TAN, C.M., XENOYANNIS, S. & FELDMAN, R.D. (1995). Oxidant stress enhances adenylyl cyclase activation. *Circ. Res.,* **77,** 710 – 717.

TESFAMARIAM, B. (1994). Free radicals in diabetic endothelial cell dysfunction. *Free Rad. Biol. Med.,* **16,** 383 – 391.

THE NATIONAL HIGH BLOOD PRESSURE EDUCATION PROGRAM WORKING GROUP. (1994). Report on hypertension in diabetes. *Hypertension,* **23,** 145 – 158.

TIETZ, N.W. (1990). *Clinical Guide to Laboratory Tests*. pp. 284 – 285. Philadelphia, Pa: WB Saunders Co.

VLASSARA, H., BUCALA, R. & STRIKER, L. (1994). Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging complications. *Lab. Invest.,* **70,** 138–151.

WEVER, R., OUDEGA, B. & VAN GELDER, B.F. (1973). Generation of superoxide radical during the autoxidation of mammalian oxyhemoglobin. *Biochim. Biophys. Acta.,* **302,** 475–478.

WILLIAMSON, J.R., TILTON, R.G., CHANG, K. & KILO, C. (1988). Basement membrane abnormalities in diabetes mellitus: relationship to clinical angiopathy. *Diabetes Metab.,* **4,** 339–370.

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