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Short communication

Pharmacological interference of vascular smooth muscle cell hypertrophy induced by glycosylated human oxyhaemoglobin

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Abstract

Nonenzymatically glycosylated human oxyhaemoglobin induces vascular smooth muscle cell hypertrophy by releasing reactive oxygen species. We analysed the ability of drugs with antihypertrophic properties for the vascular wall and/or antioxidant activity, such as captopril, losartan, and nifedipine, or gliclazide, carvedilol, and ascorbic acid, to interfere with 10 nM glycosylated human oxyhaemoglobin-induced increase in vascular smooth muscle cell size ($118 \pm 0.5\%$ of basal). Vascular smooth muscle cell hypertrophy was abolished concentration-dependently, with pD_2 values over a 100-fold interval: 6.4 ± 0.3, 7.7 ± 0.4, 7.3 ± 0.4, 7.4 ± 0.6, 8.8 ± 0.2, and 9.0 ± 0.2 for captopril, losartan, nifedipine, ascorbic acid, carvedilol and gliclazide, respectively. Drugs with powerful antioxidant properties, especially carvedilol and gliclazide, are particularly effective in preventing glycosylated human oxyhaemoglobin-induced vascular smooth muscle cell hypertrophy.

Keywords: Diabetes; Smooth muscle, vascular; Hypertrophy, cell; Oxyhaemoglobin, glycosylated; Oxidative stress

1. Introduction

Vascular alterations represent one of the most frequent complications of long-term diabetes; thus, functional and structural alterations of the vessel wall, including atherosclerosis or vascular hypertrophy, have also been observed either in patients or in animal models of the disease (Sowers and Epstein, 1995). Although the mechanisms of diabetic vascular alterations are not completely identified, the enhancement of oxidative stress in the vessel environment seems to play a key role in the development of such abnormalities (Giugliano et al., 1996). In this way, nonenzymatic glycosylation of proteins may represent an important source of reactive oxygen species (Giugliano et al., 1996). Indeed, previous work in our laboratory demonstrated that glycosylated human oxyhaemoglobin, but not non-glycosylated human oxyhaemoglobin, at concentrations in the nanomolar range, induces hypertrophy of vascular smooth muscle cells in culture (Peiró et al., 1998). This effect is mediated by the formation of different reactive oxygen species, particularly superoxide anions and hydroxyl radicals, as it can be prevented by free radical scavengers such as superoxide dismutase, deferoxamine or dimethylthiourea (Peiró et al., 1998). In the present work, we aimed to analyse the ability of different drugs with reported antihypertrophic properties for the vascular wall and/or antioxidant activity to interfere with vascular smooth muscle cell hypertrophy induced by glycosylated human oxyhaemoglobin.

2. Materials and methods

2.1. Cell culture

Primary cultures of vascular smooth muscle cells were obtained by enzymatic dissociation with collagenase of the aortae of 20-week-old Sprague–Dawley rats (Iffa-Credo,

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Labresle, France) bred at the facilities of the Facultad de Medicina Autónoma (Madrid, Spain), as previously described (Peiró et al., 1997a). Cells were characterized as vascular smooth muscle by both morphological and immunocytochemical criteria (Peiró et al., 1997a). Vascular smooth muscle cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml Amphotericin B. At confluence, cells were passaged by trypsinization with 0.05% trypsin–0.02% EDTA. Experiments were performed in vascular smooth muscle cells pooled from five animals. For experiments, vascular smooth muscle cells between passages three and ten were used.

2.2. Planar cell surface area

Planar cell surface area was determined by a previously described method (Peiró et al., 1997b). Briefly, vascular smooth muscle cells were sparsely seeded onto six-well culture plates in DMEM containing 10% foetal calf serum. After cell attachment, culture medium was switched to basal medium, i.e., serum-free DMEM supplemented with 0.1% bovine serum albumin, either alone or containing the different drugs to be tested. Vascular smooth muscle cells were cultured for 48 h with medium renewal after the first 24 h. Vascular smooth muscle cells were then fixed with 1% glutaraldehyde and planar cell surface area was quantified by computer-assisted morphometry. For this purpose, randomly selected images of vascular smooth muscle cells were transmitted to an Apple Macintosh Power 7100 computer (Cupertino, CA, USA) connected to the microscope (Nikon, Tokyo, Japan) and thereafter submitted to analysis with an appropriate software (NIH Image).

2.3. Cytotoxicity assay

The possible cytotoxic effect of the different drugs tested was assayed by quantifying the release of lactate dehydrogenase (LDH) in the cell supernatant. After the second 24-h period of treatment, supernatants were collected, centrifuged to eliminate cell debris, and assayed for LDH activity using a commercial cytotoxicity detection kit (Boehringer Mannheim, Germany). Maximum LDH release was calculated in cells lysed with 1% Triton X-100.

2.4. Materials

Culture plasticware was obtained from Costar (Cambridge, MA, USA). DMEM, foetal calf serum and trypsin-EDTA were from Biological Industries (Beit Haemek, Israel). Glycosylated oxyhaemoglobin was prepared by reduction of commercially available glycohaemoglobin (14% glycosylation; Sigma Chemical, St. Louis, MO), as previously described (Angulo et al., 1996). Ascorbic acid, and captopril were from Sigma. Nifedipine was from Bayer (Wuppertal, Germany). Losartan was a generous gift from DuPont Merck (Brussels, Belgium). Carvedilol was kindly provided by Smithkline Beecham (Collegeville, PA, USA) and gliclazide was supplied by Institut de Recherches Scientifiques Servier (IRIS, Courbevoie, France).

2.5. Statistical analysis

Values are given as mean ± S.E.M. 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 $P < 0.05$. n denotes the number of independent experiments. The value of pD_2 is defined as the negative log of the concentration required to produce half the maximum effect.

3. Results

Planar cell surface area of vascular smooth muscle cells cultured in basal conditions averaged 7356 ± 124 μm^2 ($n = 20$). After treatment of vascular smooth muscle cells with 10 nM glycosylated human oxyhaemoglobin for two consecutive periods of 24 h, planar cell surface area was enhanced to 8709 ± 44 μm^2 ($118.4 \pm 0.5\%$ of basal; $P < 0.05$, $n = 20$). This effect was not observed when vascular smooth muscle cells were treated in the same conditions with 10 nM non-glycosylated human oxyhaemoglobin ($101.3 \pm 2.7\%$ of basal planar cell surface area; $n = 10$).

In a first approach, we tested the capacity of different commonly used antihypertensive drugs, captopril, losartan and nifedipine, to inhibit the effect elicited by 10 nM glycosylated human oxyhaemoglobin. These drugs (1 nM to 10 μM) reduced the effect of glycosylated human oxyhaemoglobin in a concentration-dependent manner (Fig. 1A). For both captopril and losartan, a concentration of 10 μM was necessary to completely revert the effect of 10 nM glycosylated human oxyhaemoglobin on vascular smooth muscle cell size. In the case of nifedipine, the hypertrophic effect of glycosylated human oxyhaemoglobin was completely abolished at 1 μM, while planar cell surface area values lower than basal were observed at 10 μM (Fig. 1A). In fact, although in the absence of glycosylated human oxyhaemoglobin nifedipine had no effect by itself on cell size from 10 nM to 1 μM, it significantly reduced basal planar cell surface area at a concentration of 10 μM ($88.0 \pm 2.4\%$ of basal; $P < 0.05$, $n = 4$). On the contrary, neither captopril nor losartan did modify basal planar cell surface area by themselves ($102.3 \pm 1.4\%$ and $101.2 \pm 1.5\%$ of basal cell size at 10 μM, respectively; $n = 4$). Respective pD_2 values for captopril, losartan, and nifedipine are shown in Table 1.

In another set of experiments, different drugs with reported antioxidant activity were assayed, e.g., ascorbic acid, gliclazide, and carvedilol. These compounds inhibited the hypertrophic effect of glycosylated human oxyhaemo-

globin concentration-dependently, as shown in Fig. 1B. While gliclazide and carvedilol completely reverted the effect of glycosylated human oxyhaemoglobin at 100 nM, a 10-fold higher concentration was required for ascorbic acid. From a concentration of 100 nM, carvedilol, but not gliclazide or ascorbic acid, yielded planar cell surface area values lower than basal. When assayed on vascular smooth muscle cell cultures in the absence of glycosylated human oxyhaemoglobin, neither gliclazide nor ascorbic acid affected basal planar cell surface area by themselves (98.9 ± 3.0% and 103.5 ± 1.2% of basal, respectively; $n = 4$), while carvedilol significantly reduced cell size (85.6 ± 4.5% of basal; $P < 0.05$, $n = 3$). The values of pD_2 for ascorbic acid, gliclazide and carvedilol are shown in Table 1.

None of the tested drugs exhibited cytotoxic effects for vascular smooth muscle cells. Thus, basal LDH release was 1.75 ± 0.15% of total LDH activity ($n = 3$), while, at the highest concentration used, LDH release elicited by the different drugs did not significantly differ from basal (1.66 ± 0.08, 2.35 ± 0.08, 2.23 ± 0.71, 2.11 ± 0.78, 1.70 ± 0.65, 2.44 ± 0.61 and 2.01 ± 0.51 for 10 nM glycosylated human oxyhaemoglobin, 10 μM nifedipine, 10 μM captopril, 10 μM losartan, 1 μM gliclazide, 1 μM carvedilol and 1 μM ascorbic acid, respectively; $n = 3$).

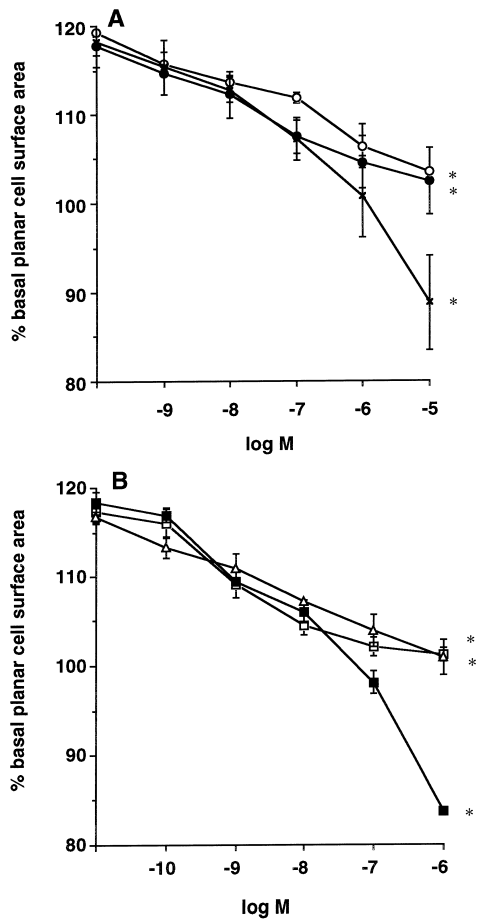

Fig. 1. Effect of (A) captopril (○), nifedipine (∗), and losartan (●), and, (B) ascorbic acid (△), gliclazide (□), and carvedilol (■), on the enhancement of vascular smooth muscle cell size induced by 10 nM glycosylated human oxyhaemoglobin. Results (mean ± S.E.M) are expressed as percentage of planar cell surface area observed in basal conditions which was of 7356 ± 124 μm^2. Data were obtained from four independent experiments. The asterisks mean a significant reduction of the increase in cell size induced by glycosylated human oxyhaemoglobin (ANOVA, $P < 0.05$).

Table 1
Values of pD_2 for the different drugs employed to inhibit the increase in cell size induced by 10 nM glycosylated human oxyhaemoglobin
Results are expressed as mean ± S.E.M. of four independent curves.

	pD_2
Captopril	6.4 ± 0.3
Nifedipine	7.3 ± 0.4
Losartan	7.7 ± 0.4
Ascorbic acid	7.4 ± 0.6
Carvedilol	8.8 ± 0.2
Gliclazide	9.0 ± 0.2

4. Discussion

Vascular hypertrophy has been implicated in the pathogenesis of diabetic vasculopathy, leading to associated hypertension and nephropathy (Sowers and Epstein, 1995). Enhanced thickness of arterial medial layer together with extracellular matrix expansion has been observed in experimental models of diabetes (Rumble et al., 1997). In addition, several in vitro studies have shown enhanced vascular smooth muscle cell growth associated with diabetes (Graier et al., 1995). Increased oxidative stress in blood vessels is a crucial event in the etiology of diabetic vascular complications (Giugliano et al., 1996). Hyperglycaemia is a key factor leading to enhanced production of reactive oxygen species by several mechanisms, such as glucose autoxidation or nonenzymatic glycosylation of proteins and advanced glycosylation end-product formation, which have been implicated in the structural vascular alterations observed in long-term diabetes (Rumble et al., 1997).

We have recently demonstrated that nanomolar concentrations of glycosylated human oxyhaemoglobin, but not non-glycosylated human oxyhaemoglobin, induce hypertrophy of cultured vascular smooth muscle cells (Peiró et al., 1998). In addition, using the ferricytochrome *c* reduction technique, we have also reported that glycosylated human oxyhaemoglobin releases higher amounts of superoxide anions than non-glycosylated human oxyhaemoglobin (Angulo et al., 1999). Our interest in oxyhaemoglobin resides in the following: (1) its degree of glycosylation is very sensitive to changes in circulating glucose levels; (2) it can be found free in plasma at low concentrations; and (3) it can penetrate into the vascular wall,

therefore reaching different vascular cell types (Rodríguez-Mañas et al., 1993). The observed hypertrophic effect of glycosylated human oxyhaemoglobin is mediated by different reactive oxygen species, particularly superoxide anions released in the extracellular space that can be converted into hydroxyl radicals acting inside vascular smooth muscle cells (Peiró et al., 1998). These data agree with those reports indicating that reactive oxygen species may modulate vascular smooth muscle cell growth, through different growth-related pathways, such as the expression of nuclear protooncogenes or the activation of mitogen-activated protein kinases (Abe and Berk, 1998).

In the present work, we analysed the possible pharmacological interference of the glycosylated human oxyhaemoglobin-induced vascular smooth muscle cell hypertrophy by using some drugs that are employed in the therapeutics of hypertension and diabetes. The angiotensin converting enzyme inhibitor captopril, the angiotensin AT_1 receptors antagonist losartan, and the Ca^{2+} channel antagonist nifedipine are antihypertensive drugs with antihypertrophic properties for cultured vascular smooth muscle cells (Peiró et al., 1997b). Indeed, both captopril and losartan reduce angiotensin II-induced vascular smooth muscle cell hypertrophy in vitro, while the Ca^{2+} channel antagonist nifedipine shows a more unspecific action, reducing cell size and protein synthesis in both hypertrophic and non hypertrophic vascular smooth muscle cells (Peiró et al., 1997b). In addition, some drugs with antioxidant properties were also tested, like gliclazide and carvedilol, while ascorbic acid was selected as reference antioxidant agent. Gliclazide is an oral hypoglycemic agent, which has been reported to have properties as general free radical scavenger (Palmer and Brogden, 1993). Carvedilol is a β-adrenoceptor antagonist, with some activity as antagonist of α1-adrenoceptors, and powerful antioxidant properties in vitro, although the clinical relevance of this fact is still not well determined (Frishman, 1998).

In the present experimental conditions, all the drugs tested were able to reduce cell size in glycosylated human oxyhaemoglobin-induced hypertrophic vascular smooth muscle cells in a concentration dependent manner. Captopril and losartan produced the weakest interference, as their maximal effects (10 μM) hardly reached basal cell size. In addition, pD_2 values for these agents were much higher than the plasmatic concentration usually required for therapeutical effects (around 230 nM for captopril and 2 nM for losartan, respectively) (Benet et al., 1996; Goa and Wagstaff, 1996). However, the inhibitory effects of these drugs lack a clear explanation, as angiotensin II is absent in this kind of in vitro vascular smooth muscle cell cultures (Peiró et al., 1997a). A possible explanation for the effect of captopril may be related to its reported actions as scavenger of free radicals when forming a captopril–iron complex (Jay et al., 1995); however, to our knowledge, there are no similar reports concerning losartan. Nifedipine exhibited a more evident antihypertrophic effect, as high concentrations of this drug were able to reduce cell size below basal values. Furthermore, the therapeutic plasmatic concentrations of nifedipine (122 ± 52 nM) (Benet et al., 1996) are in a similar range with the pD_2 value we obtained for this drug. The mechanisms involved can be related to the Ca^{2+} requirements of vascular smooth muscle cell growth processes (Jackson and Schwartz, 1992), but also to the antioxidant actions of dihydropyridines (Hishikawa and Lüscher, 1998).

Gliclazide and carvedilol also produced a concentration-dependent inhibition of glycosylated human oxyhaemoglobin-induced vascular smooth muscle cell hypertrophy. In fact, much lower concentrations of these drugs were required for antagonizing the increase in cell size, as shown by their pD_2 values. It is worth to remark that such values of pD_2 are clearly lower than the therapeutic plasmatic concentrations of these drugs (around 14 μM for gliclazide and 400 nM for carvedilol, respectively) (Palmer and Brogden, 1993; Morgan, 1994). As mentioned above, carvedilol and gliclazide possess powerful antioxidant properties (Palmer and Brogden, 1993; Frishman, 1998). However, carvedilol was able to reduce cell size even below basal, suggesting the mechanisms of action of both agents may not be exactly the same. One possibility is the capacity of carvedilol to cross the plasma membrane, which allows it to exert its action inside vascular smooth muscle cells (Yue et al., 1995). In this way, we have previously shown that hydroxyl radical scavengers acting inside vascular smooth muscle cells, such as dimethylthiourea, are much more effective in preventing glycosylated human oxyhaemoglobin-induced cell hypertrophy than hydroxyl scavengers acting only in the extracellular space (Peiró et al., 1998). Our results with carvedilol agree with previous reports concerning its capacity to prevent vascular smooth muscle cell proliferation, either in vivo or in vitro (Ohlstein et al., 1993; Patel et al., 1995), indicating that carvedilol is a powerful agent in preventing vascular smooth muscle cell growth. This effect seems to be unrelated to its properties as β-adrenoceptor antagonist or Ca^{2+} channel antagonist (Patel et al., 1995).

In conclusion, different drugs may interfere with glycosylated human oxyhaemoglobin-induced vascular smooth muscle cell hypertrophy, which is mediated by the release of reactive oxygen species. Those agents with well-known antioxidant properties, especially carvedilol and gliclazide, seem to be the most effective.

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