Enalaprilat inhibits hydrogen peroxide production by murine mesangial cells exposed to high glucose concentrations

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Abstract

Background. Oxidative stress is considered to play a role in the pathogenesis of diabetic nephropathy. Angiotensin-converting enzyme (ACE) inhibitors are hypotensive drugs with a well-known effect in preventing the progression of chronic renal failure. Their mechanism of action is not clearly established.

Methods. The effect of enalaprilat on hydrogen peroxide (H₂O₂) production by cultured murine mesangial cells exposed to 5.5 (basal condition), 30 and 50 mM glucose concentrations was examined over 8 h. A fluorimetric method quantifying, in arbitrary units, the intracellular dichlorofluorescein (DCFH) oxidation to oxygen metabolites (ROM) such as superoxide anion and hydroxyl radical are also mediators of tissue injury [3]. These reactive species are associated with both immune and non-immune glomerular injury [4,5], chronic progression of renal disease [5,6] and also with diabetic vascular complications [3,7]. However, little information exists regarding a possible role of mesangial cell in ROM production in diabetes mellitus.

Results. H₂O₂ production by mesangial cells exposed to 50 mM glucose was significantly increased after 1 h, compared to cells exposed to 5.5 and 30 mM glucose. This observation was not reproduced with 50 mM mannitol. Addition of 100 ng/ml enalaprilat to cells with 50 mM glucose significantly inhibited H₂O₂ production during the 8 h of the assay. This response was similar to that obtained with 100 ng/ml catalase. Increasing enalaprilat concentrations (10, 50 and 100 ng/ml) also significantly decreased the constitutive H₂O₂ generation in the presence of 5.5 mM glucose. Angiotensin II and saralasin, both at 1 mM, did not modify H₂O₂ production by cells exposed to 5.5 mM glucose. In contrast, 1 mM staurosporine, a protein kinase C (PKC) antagonist, significantly decreased H₂O₂ generation in the presence of 50 mM glucose.

Conclusion. Enalaprilat has an antioxidant effect in cultured mesangial cells. This action is not linked to ACE inhibition, but may be related to an inhibition of the PKC system.

Key words: enalaprilat; high glucose; hydrogen peroxide; protein kinase C; angiotensin converting enzyme; mesangial cell; diabetic nephropathy.

Introduction

Several factors (growth hormone, insulin-like growth factor-1, transforming growth factor-beta, tumour necrosis factor-alpha, interleukin-1 and others) play a role in the damaging effects of hyperglycaemia in chronic renal failure of diabetic nephropathy [1,2].

Hydrogen peroxide (H₂O₂) and other reactive oxygen metabolites (ROM) such as superoxide anion and hydroxyl radical are also mediators of tissue injury [3]. These reactive species are associated with both immune and non-immune glomerular injury [4,5], chronic progression of renal disease [5,6] and also with diabetic vascular complications [3,7]. However, little information exists regarding a possible role of mesangial cell in ROM production in diabetes mellitus.

Recently a significant lipid peroxide production in cultured rat glomeruli exposed to high glucose concentrations has been reported. The proposed biochemical pathway is a de novo synthesis of diacylglycerol resulting from hyperglycaemia, leading to an activation of the protein kinase C (PKC) enzyme [8].

Angiotensin-converting enzyme (ACE) inhibitors are drugs with significant clinical benefits in the prevention of chronic renal failure in diabetic nephropathy [9]. The mechanism of renal protection, which appears not to be fully related to their hypotensive effect [9,10], is unknown. Recently an antioxidant effect of ACE inhibitors, of interest in ROM-related diseases such as in reperfusion injury of ischaemic cardiac tissue, has been reported [11]. Moreover a possible ROM scavenging activity of these drugs in hypertensive diabetic patients has also been considered [12].

In the present study we hypothesized that an ACE inhibitor, enalaprilat, might have a modulatory effect on in vitro H₂O₂ production by cultured murine mesangial cells exposed to high glucose. The influences of angiotensin, saralasin, and staurosporine (a PKC inhibitor) were also examined in order to investigate possible involucral biochemical pathways.

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Methods

Mesangial cell isolation, culture, and characterization

Murine mesangial cells were isolated and cultured according to previous methods [13,14] with some modifications. Male Swiss mice aged 6–8 weeks were anaesthetized with intraperitoneal injection of 40 mg/kg sodium pentobarbital (nembutal). Abdominal cavity was opened and the renal vein cannulated. The abdominal aorta was incised for a retrograde perfusion of the kidney with zMEM-HEPES (Sigma Chemical Co, St Louis, Missouri, USA) at 1.5 ml/min and 37°C. Once the organ was cleared of blood, 15 ml zMEM-HEPES containing 0.07% pronase-E and 0.05% type-I collag enase were perfused at 1 ml/min and 37°C (Both enzymes were purchased from Boehringer Mannheim, Germany). The kidney was excised and the capsule eliminated. The renal parenchyma, digested by the enzymatic perfusion, was minced with two scalpels and a disaggregated suspension of renal glomeruli and tubuli was obtained. For glomerular enrichment, the suspension was consecutively pushed through 180 and 75 μm stainless steel sieves. The enriched glomerular suspension was centrifuged for 5 min at 400 r.p.m. and 4°C. The sedimented glomeruli were resuspended and cultured in plastic tissue culture flasks (Corning Corp., Corning, New York, USA) with bicarbonate-buffered zMEM, containing 5.5 mM glucose, 92 mg/ml D-valine, which inhibited the growth of contaminating fibroblasts, 100 μ/ml penicillin, 100 μg/ml streptomycin (tissue culture media and antimicrobials provided by Sigma Chemical Co, St Louis, Missouri, USA), and 10% fetal calf serum (Gibco BRL, Egg enstein, Germany). Flasks were incubated in a 5% CO₂ humidified atmosphere at 37°C. Culture media were changed every 2–3 days and subcultures plated every 1–2 weeks, using 1% trypsin plus 10 mM EDTA in PBS (all provided by Sigma Chemical Co, St Louis, Missouri, USA).

By the third subculture the predominant cell type had a mesangial cell phenotype. The cells were stellate-shaped and immunolabelling experiments with monoclonal antibodies specific for iso-a-smooth-muscle actin and desmocollin were positive, and those for von Willebrand factor-VIII and 18-cytokeratin were negative. In vitro endocytosis assays using FITC-conjugated ovalbumin (100 ng/ml for 2 h), acetylated-low density lipoprotein (10 mg/ml for 4 h) and 1 μm-sized FITC-conjugated latex particles (1 x 10⁹ particles/ml, for 1 h) were also negative. All these results suggest the lack of contaminating macrophages and epithelial and endothelial cells. We also evaluated the homogeneity of the mesangial cell cultures by flow cytometry with immunolabelling with iso-a-smooth-muscle actin.

In vitro treatment conditions of mesangial cells

Homogeneous cultures were obtained after more than 10 subcultures. Then mesangial cells were resuspended and sedimented on 24-well plates. When confluent cultures were reached, cells were exposed to 3.5 (basal), 30 and 50 mM glucose for 8 h. In comparative experiments 10, 50 and 100 ng/ml enalaprilat, soluble active form of the orally administered ACE inhibitor enalapril® (Merck Sharp & Dohme, Madrid, Spain) were added to the three studied glucose concentrations. In other assays we tested control groups of mesangial cells in the presence of mannitol (added to 5.5 mM glucose to obtain a 50 mM solution). Catalase (100 ng/ml) was added to the controls for the study of ROM scavenger activity.

In a second study we examined the influence of angiotensin II and saralasin on cells exposed to basal glucose concentrations, and of stauroporine on cells in the presence of elevated glucose. The three peptides (provided by Sigma Chemical Co, St Louis, MO) were evaluated at 1 nM concentration.

Measurement of in vitro hydrogen peroxide production by mesangial cells

On the day of the assay, fresh HEPES-buffered zMEM culture medium without phenol red was exchanged and basal autofluorescence was determined using a CytoFluor-2350 system (Millipore Co., Bedford, Massachusetts, USA) at 485/22 nm excitation with a 530/25 nm emission filter at high and intermediate sensitivity settings. H₂O₂ production was evaluated by microfluorimetry, with a method reported for other cell lineages [15,16] and, to our knowledge, not for cultured mesangial cells.

A probe, dichlorofluorescein-diacetate (DCFH-DA) (20 mM), was incubated with mesangial cells for 20 min and washed before addition of experimental conditions. With this method the H₂O₂ production is evaluated by the intracellular dichlorofluorescin (DCFH) oxidation to the highly fluorescent compound 27′dichlorofluorescein (DCF) from the non-fluorescent probe DCFH-DA. Fluorescence was recorded at 60-min intervals over 8 h in arbitrary fluorescence units after subtracting autofluorescence. Each assay was repeated three times and all experiments were performed in quadruplicate wells.

Statistical evaluation

The results were expressed as mean ± standard deviation. Data were analysed by Student’s two-tailed, unpaired t test. Analysis of variance (ANOVA) was also applied when multiple mean comparison was required. P values less than 0.05 were used as the criterion for statistically significant difference.

Results

Effect of glucose concentrations on hydrogen peroxide production

Figure 1 shows the results of the fluorometry experiments measuring intracellular conversion of DCFH to DCF over 8 h, which represents in vitro H₂O₂ production by mesangial cells exposed to basal (5.5 mM) and high glucose (30 and 50 mM) concentrations. A sustained hourly increase in this H₂O₂ generation was observed during all 8 h of observation for every glucose concentration.

The H₂O₂ production in the presence of 30 mM did not significantly differ from that with 5.5 mM at any time. In the presence of 50 mM glucose, H₂O₂ production was significantly increased beginning at the first hour (P < 0.05, higher than twofold) with respect to cells exposed to the other glucose concentrations. This response was maintained with even more significant results from the second hour to the end of the observations (P < 0.01).

Figure 2 shows the H₂O₂ production in mesangial cells exposed to 50 mM mannitol, a solution with an...
Fig. 1. Effect of increasing glucose concentrations on \textit{in vitro} \textit{H}_2\textit{O}_2 production by cultured mesangial cells. Cells were incubated at 37°C with 20 mM DCFH-DA for 20 min and washed. Then they were exposed to 5.5, 30 and 50 mM glucose for 8 h. Plate-scanning fluorimetric recording of intracellular DCFH oxidation to the highly fluorescent compound DCF was proportional to \textit{H}_2\textit{O}_2 production. The DCF fluorescence intensity was measured in fluorescence arbitrary units. Data represent average values ± SD of quadruplicate determinations in three separate experiments. Differences were statistically significant in the case of 50 mM glucose treated cells compared with 5.5 and 30 mM. (#P < 0.05, ##P < 0.01). The hourly variations were also significantly higher in the case of 50 mM glucose (**P < 0.001). Statistical analysis was performed by Student’s two-tailed unpaired \textit{t} test and ANOVA for multiple mean comparison.

Fig. 2. Effect of physiological (5.5 mM) and elevated (50 mM) glucose concentrations on \textit{in vitro} \textit{H}_2\textit{O}_2 production by cultured mesangial cells, comparing with the influence of a 50 mM mannitol solution. The methodologies of measurement, registry and statistical analysis are identical to those described in Figure 1. In the presence of 50 mM mannitol, the \textit{H}_2\textit{O}_2 production did not differ from that obtained with 5.5 mM glucose. In contrast, \textit{H}_2\textit{O}_2 production with elevated glucose was significantly higher than with mannitol (#P < 0.05, ##P < 0.01) 50 mM glucose compared to 50 mM mannitol at every time point; *P < 0.01, **P < 0.001 every value compared to control of time 0).

equivalent osmotic power to that of 50 mM glucose. There was also a significant \textit{H}_2\textit{O}_2 production (P < 0.01 at first hour and P < 0.001 at the eighth hour on comparing with the control of time 0). However, these determinations did not significantly vary with respect to those obtained under 5.5 mM glucose. Moreover, the \textit{H}_2\textit{O}_2 values under 50 mM glucose were statistically more significant than those recorded under 50 mM mannitol (P < 0.05 at first h and P < 0.01 at eighth hour).
Enalaprilat inhibits glucose-derived H$_2$O$_2$ production by cultured mesangial cells

**Effect of enalaprilat on glucose-induced hydrogen peroxide production by mesangial cells**

Figure 3 summarizes the recordings of *in vitro* H$_2$O$_2$ generation by cells under basal glucose conditions when exposed to three increasing concentrations of enalaprilat (10, 50 and 100 ng/ml). A statistically significant decrease in H$_2$O$_2$ production was evident for all cases. The results were more significant with 50 and 100 ng/ml. The constitutive H$_2$O$_2$ production at 8 h (near sevenfold the value of time 0) was reduced to nearly threefold the baseline in the presence of both enalaprilat concentrations ($P<0.01$). H$_2$O$_2$ synthesis was diminished by a lesser degree (only to fourfold of baseline) with 10 ng/ml enalaprilat at the 8th hour ($P<0.05$).

The treatment of cells exposed to high glucose (30 and 50 mM) with 100 ng/ml enalaprilat also inhibited H$_2$O$_2$ production. These observations are shown in Figures 4 and 5. In both cases, recordings were decreased with statistically significant results beginning with the first hour ($P<0.05$) and this effect was maintained, or even more evident ($P<0.01$), over the 8 h of the assays compared to non-treated cells.

Figure 6 shows the H$_2$O$_2$ production in the presence of 50 mM glucose obtained with the addition of 100 ng/ml enalaprilat and that detected with 100 ng/ml catalase. The results with the ACE inhibitor very nearly reproduced the inhibitory effect on H$_2$O$_2$ production that was characteristic of the ROM scavenger, although this effect was less intense at the end of the observation period (catalase-treated versus enalaprilat-treated cells at the 8th hour, $P<0.05$).

**Effect of angiotensin II and saralasin on cells exposed to glucose basal concentration**

Figures 7 and 8 depict the H$_2$O$_2$ production of cultured mesangial cells maintained in basal 5.5 mM glucose exposed to angiotensin II and its competitive inhibitor, saralasin, both at 1 mM concentration. The H$_2$O$_2$ generation, evaluated for the two peptides, both at the start and at the end of the observation period did not significantly vary compared to non-treated cells.

**Effect of staurosporine on cells exposed to elevated glucose concentration**

Figure 9 shows that the mesangial H$_2$O$_2$ production in the presence of 1 mM staurosporine exhibited a statistically significant decrease ($P<0.01$ at the first hour and at the end of the observation period compared to non-treated cultures) in 50 mM glucose concentration.

The inhibition on H$_2$O$_2$ production was not so intense as that observed in catalase-treated cells ($P<0.01$ catalase versus staurosporine both at the first and at the eighth hour). Statistical analysis also showed significant differences between the effects of staurosporine and enalaprilat on H$_2$O$_2$ production ($P<0.01$ ACE inhibitor versus the PKC inhibitor, both at the first and at the eighth hour of observation).

**Discussion**

Diabetic nephropathy is a form of chronic renal failure characterized by glomerular hypertrophy with basement membrane thickening, decreased intraglomerular cellularity and increased deposition of mesangial matrix [1]. The mechanism by which hyperglycaemia leads to glomerular mesangial damage remains unclear [2]. Glucose-induced oxidative stress has recently been proposed as playing a role in vascular complications and chronic renal damage in diabetes mellitus [3, 7, 8, 17].
Fig. 4. Inhibitory effect of enalaprilat on \textit{in vitro} \(\text{H}_2\text{O}_2\) production by mesangial cells exposed to high glucose concentration. The \(\text{H}_2\text{O}_2\) production by mesangial cells exposed to 30 mM glucose plus 100 ng/ml enalaprilat was compared to cells receiving only medium containing 30 mM glucose for 8 h. Results are expressed as percent variations at any time during exposure over the 100% at time 0. Data represent average values \(\pm\) SD of quadruplicate determinations in three separate experiments. Differences were statistically significant by Student’s two-tailed unpaired \(t\) test for the enalaprilat-treated cells with respect to non-enalaprilat-treated mesangial cells beginning at the first hour (*\(P<0.05\), **\(P<0.01\) on comparing every time point).

Fig. 5. Inhibitory effect of enalaprilat on \textit{in vitro} \(\text{H}_2\text{O}_2\) production by mesangial cells exposed to high glucose concentration. The \(\text{H}_2\text{O}_2\) production by mesangial cells exposed to 50 mM glucose plus 100 ng/ml enalaprilat was compared to cells receiving only medium containing 50 mM glucose for 8 h. Results are expressed as in Figure 4. Differences were statistically significant by Student’s two-tailed unpaired \(t\) test for the enalaprilat-treated cells with respect to non-enalaprilat-treated mesangial cells beginning at the first hour (*\(P<0.05\), **\(P<0.01\) on comparing every time point).

The mesangium might be another source of ROM in the presence of metabolic disturbances. However, the hypothetical link between hyperglycaemia and oxidative stress in mesangium has not been extensively explored. Recently, elevated levels of lipid peroxides, an indirect index of increased oxidative stress and subsequent cytotoxicity, have been observed in cultured rat glomeruli under high glucose concentrations [8]. In this study we demonstrated a glucose-dependent \(\text{H}_2\text{O}_2\) production by cultured mesangial cells using microfluorimetry, a method not previously employed for this cell type. A sustained hourly increase was observed for three glucose
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Fig. 6. Inhibitory effect of enalaprilat on in vitro H$_2$O$_2$ production by mesangial cells exposed to high glucose concentration comparing with the effect observed in a catalase-treated control group. The methodologies of measurement, registry, and statistical analysis are identical to those described in Figure 1. During an observation period of 8 h, cells exposed to 50 mM glucose plus and without 100 ng/ml enalaprilat were compared with another group of cells exposed to 50 mM glucose with 100 ng/ml catalase. The inhibitory effect of enalaprilat was very similar to that obtained by the antioxidant action of catalase, although it was less intense at 8 h (*P<0.05 on comparing enalaprilat-treated with respect to catalase-treated cells).

Fig. 7. Evaluation of angiotensin II on in vitro H$_2$O$_2$ production by mesangial cells. The methodologies of measurement, registry, and statistical analysis are identical to those described in Figure 1. Responses in the presence of 5.5 mM glucose, with and without 1 mM angiotensin II, were compared to a group of cells exposed to only 50 mM glucose (*P<0.01, **P<0.001 with respect to control of time 0). There was neither a significant stimulatory, nor an inhibitory effect on angiotensin-II-treated cells.

concentrations (5.5, 30 and 50 mM) over a period of 8 h. Lack of effect of 50 mM mannitol suggested that H$_2$O$_2$ production was clearly independent from a high media osmolarity influence per se.

The lack of difference between basal, physiological 5.5 mM glucose and 30 mM, in contrast with the more significant hyperproduction achieved with 50 mM glucose suggest that extreme hyperglycaemia, even for a short period, might have a critical role in the development of glomerular damage. However, we must consider that mesangial cells are located in the centre of glomeruli. Thus the glucose concentration in the pericellular or intracellular space may not be the same...
Fig. 8. Evaluation of saralasin on in vitro H$_2$O$_2$ production by mesangial cells. The methodologies of measurement, registry, and statistical analysis are identical to those described in Figure 1. Responses in the presence of 5.5 mM glucose, with and without 1 mM saralasin, were compared to a group of cells exposed to only 50 mM glucose (**P<0.01, **P<0.001 with respect to control of time 0). There was neither a significant stimulatory, nor an inhibitory effect on saralasin-treated cells.

Fig. 9. Evaluation of staurosporine on in vitro H$_2$O$_2$ production by mesangial cells. The methodologies of measurement, registry, and statistical analysis are identical to those described in Figure 1. Responses in the presence of 50 mM glucose, with and without 1 mM staurosporine, were compared to a group of cells exposed to 50 mM glucose plus 100 ng/ml enalaprilat and another group of cells exposed to 100 ng/ml catalase. A statistically significant inhibition of H$_2$O$_2$ production in the presence of staurosporine was observed (*P<0.01 versus non-treated cells). These results did not fully reproduce the effect of catalase (**P<0.01 on comparing staurosporine to catalase values) nor the effect of enalaprilat (#P<0.01 on comparing staurosporine to enalaprilat values).

as in the peripheral circulating levels. In addition, murine mesangial cells may behave differently than those of human origin.

Once originated, and if not efficiently scavenged, H$_2$O$_2$, as any ROM, may cause tissue damage by well-known pathological mechanisms: oxidation of lipid cellular components (cytoplasmic membranes), peroxidation of lipoproteins, and denaturation of protein molecular structures. These injuries could account for the vascular damage seen in atherosclerosis and diabetic microangiopathy [3–5]. In particular, mesangial extracellular matrix and glomerular basement membrane degeneration could
correlate with ROM generation and with chronic glomerular damage [6,7].

ACE inhibitors are potent drugs employed to control hypertension. Recent controlled trials have shown a significant benefit of these drugs in the prevention of chronic renal failure related to diabetic nephropathy and other chronic renal diseases [9]. Their renal protection was initially attributed to blood pressure reducing effect, resulting in improved intrarenal haemodynamics and decreased intraglomerular hyperfiltration. However, a protective action not linked with their hypotensive effect has also been observed in human and experimental studies [9,10].

Recently some authors have reported protective effects of ACE inhibitors on experimental myocardial and vascular damage after oxidative stress [11,12]. Initially, the scavenging activity was thought to be related to sulphydryl-containing drugs such as captopril, but protective properties of other non-sulphhydryl-containing ACE inhibitors have also been reported [12]. The exact explanation for the mechanism of antioxidant action of these molecules remains unclear.

Our data have confirmed a protective effect of one non-sulphhydryl-containing drug, enalaprilat, the soluble, active form of the oral administered ACE inhibitor enalapril, on in vitro H$_2$O$_2$ production by mesangial cells. At basal and high glucose concentrations, this drug significantly diminished H$_2$O$_2$ production in a catalase-like action, although the amount of inhibition with catalase was more extreme than that obtained with enalaprilat. Even at the very low concentration of 10 ng/ml, this drug showed significantly a negative modulating effect over the constitutive H$_2$O$_2$ production by cells under basal glucose.

Angiotensin II plays a role in diabetic nephropathy and other forms of chronic renal damage by influencing extracellular matrix synthesis in an autocrine or paracrine fashion via its haemodynamic and mitogenic actions [18–20]. Thus, a reduction of the intrarenal levels of this peptide might contribute to renal protection [18]. However, our results suggest that the antioxidant effect of enalaprilat is not linked with ACE inhibition.

We used staurosporine to investigate an alternative biochemical pathway, the PKC system. In the presence of 50 mM glucose, H$_2$O$_2$ production was significantly decreased after addition of this potent PKC inhibitor. Thus, a renal protection based on antioxidant properties and the inhibition of the PKC system was strongly suggested with our results. In agreement with this observation, some authors have reported that hyperglycaemia can lead to renal damage via de novo synthesis of diacylglycerol and subsequent PKC activation [8]. Nevertheless, the degree of inhibition of H$_2$O$_2$ production achieved with staurosporine and enalaprilat in the presence of 50 mM glucose was significantly different. Additionally, the inhibition with staurosporine did not fully reproduce the effect of catalase. Thus, we think these data do not exclude the involvement of other mechanisms different from inhibition of PKC in the antioxidant action of enalaprilat.

To our knowledge, this antioxidant property is not described for any ACE inhibitor on cultured mesangial cells. Although other ACE inhibitors were not examined in this work, we think that the beneficial effect of these drugs on delaying the progression of renal failure might be based, at least partially, on antioxidant effects linked to an inhibition of the PKC system.

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Abbreviations

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<th>Acronym</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
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<td>CO$_2$</td>
<td>Carbonic anhydride</td>
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<td>Reactive oxygen metabolites</td>
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<td>SD</td>
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