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Captopril and quinapril reduce reactive oxygen species

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Abstract

Background Angiotensin-converting enzyme inhibitors may affect reactive oxygen species in humans *in vitro* and *in vivo*. In the present study we evaluated whether angiotensin-converting enzyme inhibitors may affect NAD(P)H oxidase activity.

Materials and methods The production of reactive oxygen species was measured spectrophotometrically in mononuclear leukocytes using the fluorescent dye, dichlorofluorescein diacetate. The effects of quinaprilat, captopril, enalaprilat and lisinopril on phorbol myristate acetate-induced reactive oxygen species generation were investigated *in vitro*. The effects of quinaprilat, captopril, enalaprilat and lisinopril on the NAD(P)H oxidase activity of the mononuclear leukocytes were measured photometrically. In addition, reactive oxygen species were measured before and 4 h after oral administration of quinapril.

Results *In vitro*, the addition of quinaprilat ($72 \pm 6\%$ of control; mean ± SEM; $n = 19$; $P < 0{\cdot}001$) and captopril ($48 \pm 2\%$ of control; $n = 19$; $P < 0{\cdot}001$) significantly reduced the phorbol-12-myristate-13-acetate-induced reactive oxygen species generation by the mononuclear leukocytes, whereas enalaprilat and lisinopril showed no effect. The effect of captopril on phorbol-12-myristate-13-acetate-induced reactive oxygen species generation *in vitro* was concentration-dependent. Quinaprilat and captopril significantly inhibited the NAD(P)H oxidase activity. After the oral administration of 10 mg of quinapril the phorbol-12-myristate-13-acetate-induced reactive oxygen species generation by the mononuclear leukocytes was significantly decreased from $1981 \pm 292\%$ to $988 \pm 141\%$ ($n = 14$; $P < 0{\cdot}01$).

Conclusion Quinapril and captopril decrease the production of reactive oxygen species.

Keywords Angiotensin converting enzyme inhibitor, fluorescent dye, mononuclear leukocytes, NAD(P)H oxidase, reactive oxygen species.
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Introduction

Reactive oxygen species (ROS) are by-products of physiological metabolism in cells, are generated after cell stimulation, and interact with intracellular signal transduction [1–4]. The generation of the superoxide anion by mononuclear leukocytes is involved in host defense and in the pathogenesis of hypertension and atherosclerotic vascular disease [5–7]. Enhanced superoxide production has been shown in various animal models of hypertension or atherosclerosis [8–11]. In the oxidative-modification hypothesis of atherosclerosis it is proposed that oxidation of lipids in low density lipoproteins is an early step in the pathogenesis of atherosclerosis. In addition, epidemiological studies have linked antioxidative treatment with a reduction in the clinical manifestations of atherosclerosis [12].

The major source of reactive oxygen species in mononuclear leukocytes is NAD(P)H oxidase. NAD(P)H oxidase contains two membrane-bound components, p22phox and gp91phox, and several cytoplasmic subunits, p47phox, p67phox, and rac [5,6,13]. In neutrophils, NAD(P)H oxidase activity is stimulated after activation of protein kinase C [13,14]. In other cells, NAD(P)H oxidase activity is stimulated by several growth factors and hormones, including platelet-derived growth factor, tumour necrosis factor-α and angiotensin II. Angiotensin II increases NAD(P)H-related superoxide production in vascular smooth muscle cells and glomerular mesangial cells [15,16].

Angiotensin-converting enzyme inhibitors are known to have beneficial effects on cardiovascular morbidity and mortality [17–19]. Therefore, it may be speculated that some of the beneficial effects of angiotensin-converting enzyme

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inhibitors might be a result in part of their antioxidative capacity. Here, we investigated the effect of angiotensin-converting enzyme inhibitors on the generation of reactive oxygen species by mononuclear leukocytes.

Methods

The effects of angiotensin-converting enzyme inhibitors on the generation of reactive oxygen species were measured in mononuclear leukocytes from 19 healthy subjects (mean age: 60 ± 3 years; body mass index 26 ± 1 kg m^{-2}) without any medication *in vitro*. In addition, we investigated 14 essential hypertensive patients (mean age: 64 ± 3 years; body mass index 28 ± 2 kg m^{-2}). Age ($P = 0{\cdot}53$) or body mass index ($P = 0{\cdot}10$) were not significantly different between the two groups. In the patients with essential hypertension, blood was obtained before and 4 h after the oral administration of quinaprilat 10 mg. The study was approved by the local Ethics Committee and informed consent was obtained from each patient. None of the subjects was on NSAID, vitamin E or other antioxidants.

Measurement of ROS generation

Mononuclear leukocytes were isolated from the heparinized blood samples according to a previously described procedure [20]. Reactive oxygen species generation in the mononuclear leukocytes was measured using fluorescence spectrophotometry [20]. The mononuclear leukocytes were incubated with the dye, 2′,7′-dichlorofluorescin diacetate (DCF-DA, 5 μmol L^{-1}), for 15 min and then washed and resuspended in physiological salt solution. By using DCF-DA, intracellular ROS are determined. It is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and is thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [20]. The level of DCF fluorescence, reflecting the concentration of ROS, was monitored spectrophotometrically (F-2000 Hitachi, Tokyo, Japan) at 534 nm after excitation at 488 nm. The mononuclear leukocytes were stimulated with 1 μmol L^{-1} phorbol-12-myristate-13-acetate (PMA) or 200 nmol L^{-1} N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and the percent fluorescence increase was determined after 30 min. The cells were stimulated with PMA in the absence and presence of angiotensin-converting enzyme inhibitors [quinaprilat (which is the active metabolite of quinapril *in vivo* and was kindly provided by Gödecke–Parke-Davis, Freiburg, Germany), captopril, enalaprilat or lisinopril (Sigma Chemical Co., St. Louis, MO, USA), or the specific inhibitor of NAD(P)H oxidase diphenylene iodonium (DPI; Calbiochem, La Jolla, CA, USA)] [10]. Angiotensin-converting enzyme inhibitors or DPI were added immediately before assaying. The angiotensin-converting enzyme inhibitors alone did not show fluorescence or quenching of DCF fluorescence. For maximal stimulation a PMA concentration of 1 μmol L^{-1} was used in accordance with previous studies. The concentration-dependent effect of PMA on ROS generation has recently been described [21].

Measurement of NAD(P)H oxidase activity

Cell membranes from the mononuclear leukocytes were obtained after freeze-thawing cycles and incubated with 5 mmol L^{-1} NADH in the absence or presence of quinaprilat, captopril, enalaprilat, lisinopril or DPI. Angiotensin-converting enzyme inhibitors or DPI were added immediately before assaying. The decrease of the absorbance of NADH at 340 nm within 60 min was measured. The changes in the absorbance obtained after 60 min in the presence of angiotensin-converting enzyme inhibitors or DPI were compared with the changes obtained under control conditions. Use of DPI, a specific inhibitor of NAD(P)H oxidase activity, confirmed that under these experimental conditions the decrease of NADH absorbance was the result of The NAD(P)H oxidase activity. Under control conditions the activity of the NAD(P)H oxidase was set to 100%. The angiotensin-converting enzyme inhibitors alone did not show absorbance at 340 nm.

Statistics

Data are means ± SEM. Differences of the effects of treatment were compared with the Wilcoxon Mann–Whitney test for paired data, using the computer software GraphPad Prism 3·0 (GraphPad Software, San Diego, CA, USA). Two-tailed values of $P < 0{\cdot}05$ were considered significant. Where error bars do not appear in the figures, the error was within the size of the symbol.

Results

The inhibitory effect of angiotensin-converting enzyme inhibitors on ROS generation *in vitro* is shown in Fig. 1. The original tracings indicate that in the presence of captopril the PMA-induced fluorescence increase in the mononuclear leukocytes loaded with the ROS-sensitive dye, 2′,7′-dichlorofluorescein, was decreased. Under control conditions the PMA-induced ROS generation *in vitro* was set to 100%. Compared with the control conditions the addition of quinaprilat (72 ± 6% of control; $n = 19$; $P < 0{\cdot}001$) and captopril (48 ± 2% of control; $n = 19$; $P < 0{\cdot}001$) significantly decreased the PMA-induced ROS generation in the mononuclear leukocytes (Fig. 1b). On the other hand, the addition of enalaprilat (100 ± 7% of control; $n = 19$) and lisinopril (92 ± 6% of control; $n = 19$) did not significantly decrease the PMA-induced ROS generation in the mononuclear leukocytes. The effects of angiotensin-converting enzyme inhibitors on PMA-induced ROS generation were compared with the effect of the specific NAD(P)H oxidase

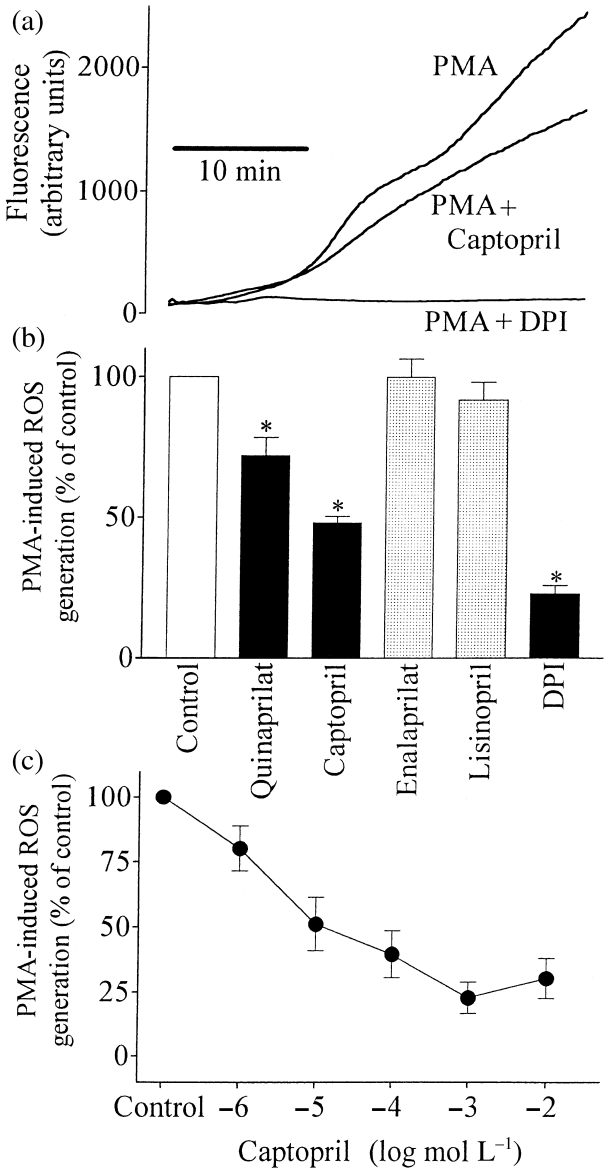

Figure 1 Effect of angiotensin-converting enzyme inhibitors on the phorbol-12-myristate-13-acetate (PMA)-induced generation of reactive oxygen species (ROS). (a) Original tracings showing the fluorescence increase of mononuclear leukocytes loaded with the ROS-sensitive fluorescent dye 2′,7′-dichlorofluorescein diacetate after addition of 1 μmol L^{-1} PMA in the presence of 10 μmol L^{-1} captopril or in the presence of the specific inhibitor of the NAD(P)H oxidase, diphenylene iodonium (DPI, 10 μmol L^{-1}). (b) Bar graph showing the effects of quinaprilat, captopril, enalaprilat, lisinopril (final concentrations, 10 μmol L^{-1}) and DPI (final concentration 10 μmol L^{-1}) on PMA-induced ROS-generation in mononuclear leukocytes. After 30 min the PMA-induced fluorescence increase was determined. The PMA-induced fluorescence increase under control conditions was set to 100%. $^{*}P < 0{\cdot}001$ compared with control conditions. (c) Line graph showing the concentration-dependent effect of captopril on PMA-induced generation of reactive oxygen species. Data are mean ± SEM from six experiments.

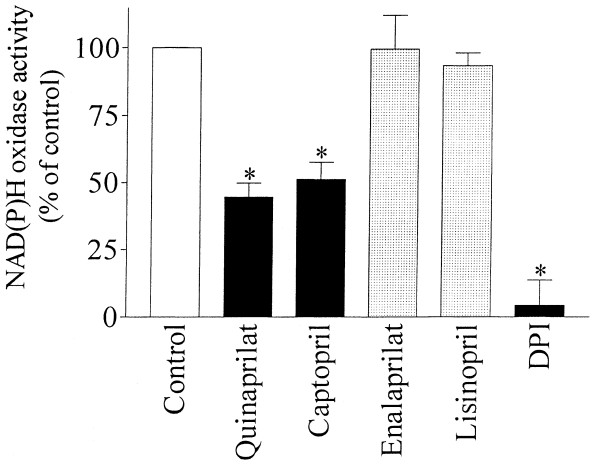

Figure 2 Effect of angiotensin-converting enzyme inhibitors on the NAD(P)H oxidase activity of mononuclear leukocytes. Bar graph showing the effects of quinaprilat, captopril, enalaprilat, lisonopril (final concentrations 10 μmol L^{-1}) or DPI (final concentration 10 μmol L^{-1}) on the NAD(P)H oxidase activity. The changes in absorbance obtained after 60 min in the presence of angiotensin-converting enzyme inhibitors or DPI were compared with the changes obtained under control conditions. Under control conditions NAD(P)H oxidase activity was set to 100%. Data are mean ± SEM from eight experiments. $^{*}P < 0{\cdot}001$ compared with control conditions.

inhibitor, diphenylene iodonium (Fig. 1). In the presence of diphenylene iodonium the PMA-induced ROS generation in the mononuclear leukocytes was significantly decreased to 23 ± 3% of control ($n = 8$; $P < 0{\cdot}001$). Captopril also significantly reduced the fMLP-induced ROS generation in the mononuclear leukocytes to 70 ± 6% of the control ($n = 3$, $P < 0{\cdot}05$).

As shown in Fig. 1(c) the inhibitory effect of captopril on PMA-induced ROS generation *in vitro* was concentration-dependent. With captopril concentrations as low as 1 μmol L^{-1} a significant inhibition of ROS production was observed ($P < 0{\cdot}01$). The maximal effect was reached with 1 mmol L^{-1}.

The activity of the major ROS generating enzyme, NAD(P)H oxidase, was measured photometrically. The inhibitory effect of angiotensin-converting enzyme inhibitors on the NAD(P)H oxidase activity of the mononuclear leukocytes is shown in Fig. 2. Under control conditions the activity of the NAD(P)H oxidase was set to 100%. The addition of the specific inhibitor of NAD(P)H oxidase, diphenylene iodonium, completely inhibited that enzyme. Compared to control conditions the addition of quinaprilat (45 ± 5% of control; $P < 0{\cdot}001$) and captopril (51 ± 6% of control; $P < 0{\cdot}001$) significantly reduced the activity of the NAD(P)H oxidase of mononuclear leukocytes (Fig. 2). On the other hand, the addition of enalaprilat (100 ± 13% of control) and lisinopril (93 ± 5% of control) did not significantly decrease the activity of the NAD(P)H oxidase of mononuclear leukocytes.

Next the effect of quinaprilat on ROS was evaluated in hypertensive patients *in vivo*. Four hours after the oral

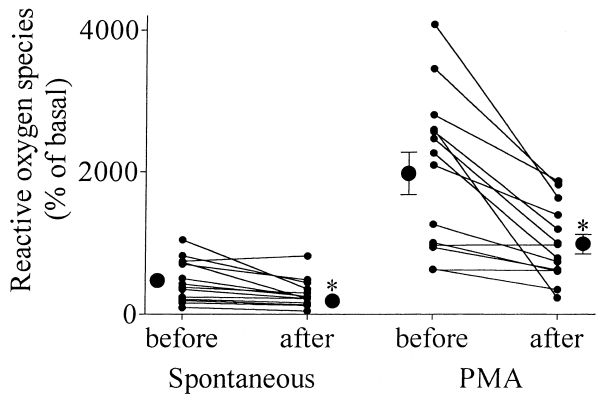

Figure 3 Reactive oxygen species (ROS) generation before and 4 h after oral administration of quinapril 10 mg. The spontaneous and phorbol-12-myristate-13-acetate (PMA)-induced generation of ROS in mononuclear leukocytes from 14 subjects were measured spectrophotometrically using the fluorescent dye 2′,7′-dichlorofluorescein diacetate. Resting 2′,7′-dichlorofluorescein fluorescence was set to 100%. The spontaneous and PMA-induced fluorescence increase was measured. Individual data and mean ± SEM values are given. $^{*}P < 0{\cdot}01$ for comparison before and after the oral administration of quinapril.

administration of 10 mg quinapril the spontaneous ROS generation was significantly reduced from 468 ± 77% to 286 ± 51% ($n = 14$; $P < 0{\cdot}01$). Four hours after the oral administration of 10 mg quinapril the PMA-induced ROS generation in mononuclear leukocytes was significantly reduced from 1981 ± 292% to 988 ± 141% ($n = 14$; $P < 0{\cdot}01$). Figure 3 shows the individual data for each subject. Four hours after the oral administration of quinapril the systolic/diastolic blood pressure was significantly reduced from 154 ± 8/89 ± 5 mmHg to 133 ± 7/77 ± 4 mmHg ($P < 0{\cdot}01$).

In healthy control subjects without administration of quinapril the spontaneous ROS generation was 512 ± 113% and 562 ± 26% four hours later, showing no significant differences. In control subjects without administration of quinaprilat the PMA-induced ROS generation in mononuclear leukocytes was 1238 ± 338% and 1497 ± 557% four hours later, showing no significant differences. The PMA-induced ROS generation in patients before quinapril administration (1981 ± 292%) was not significantly different to the control subjects (1238 ± 338%; $P = 0{\cdot}28$).

Discussion

The results document that the angiotensin-converting enzyme inhibitors, quinapril and captopril, inhibit the production of ROS *in vitro*, whereas enalaprilat and lisinopril showed no effect. One underlying mechanism appears to be the inhibition of NAD(P)H oxidase in mononuclear leukocytes. The inhibition of NAD(P)H oxidase is observed using membrane-associated NAD(P)H oxidase in disrupted cells. However, interference with signalling pathways or the assembly of an NAD(P)H oxidase enzyme complex might also be considered.

Some authors have reported on the specific properties of angiotensin-converting enzyme inhibitors [22–26]. DeCavanagh *et al.* studied the effect of enalapril and captopril on total glutathione content as well as glutathione peroxidase and reductase activation in mouse tissue [22]. Total glutathione content increased in several tissues after administration of enalapril or captopril, and both glutathione peroxidase and reductase activities were enhanced. In red blood cells, as a net effect, oxidative stress was decreased by these angiotensin-converting enzyme inhibitors, and NO production was increased, possibly due to decreased peroxynitrite formation. Furthermore, in murine lung alveolar macrophages, ROS production after stimulation with PMA was inhibited by the angiotensin-converting enzyme inhibitor, alacepril [23]. These results are similar to the effects of quinaprilat and captopril in human mononuclear leukocytes. Hayek *et al.* examined the effect of captopril in the presence of low density lipoproteins. The authors further observed an antiatherosclerotic effect of captopril in apolipoprotein E-deficient mice [24]. That effect might be related to the direct antioxidative effect of captopril on low density lipoproteins. Using a ferric reducing power assay, Benzie & Tomlinson [25] found that only captopril, but none of the other angiotensin-converting enzyme inhibitors tested, had direct antioxidative power. Similar effects of captopril, but not enalapril, were observed in a cell-free assay system by Bartosz *et al.* [26].

Clapperton *et al.* [27] measured the effect of captopril on stimulated neutrophils using chemiluminescence. These authors showed that captopril caused an initial delay in luminol chemiluminescence production by PMA-stimulated neutrophils, whereas NAD(P)H oxidase was not affected. These data are somewhat different from the present results obtained from the intracellular fluorescence measurements of the reactive oxygen species the in mononuclear leukocytes and from the measurements of the NAD(P)H oxidase activity. The differences may be because of the different methodologies and cell types used. In contrast, our findings are in accordance with earlier reports in showing the specific effects of captopril. Beyond these findings, captopril and quinapril appear to exert significant antioxidative effects also in human cells and in patients taking these angiotensin-converting enzyme inhibitors in the recommended dosage. Our results further indicate that the decrease in reactive oxygen species aroused by the angiotensin-converting enzyme demonstrable *in vitro* may be relevant in humans treated with certain angiotensin-converting enzyme inhibitors in usual doses. The effect observed *in vivo* after administration of quinapril may contribute to the vasodilator effects of angiotensin-converting enzyme inhibitors and could also exert antiatherosclerotic effects similar to those seen in animal experiments [24]. As angiotensin-converting enzyme inhibitors show several structural differences, it can only be speculated which structure might be responsible for the observed effects.

In our *in vitro* studies concentrations were used that are similar to the plasma concentrations of angiotensin-converting enzyme inhibitors obtained *in vivo* during their therapeutic use. Therapeutic plasma concentrations from 1 to 10 μmol L^{-1} had been reported for captopril or quinaprilat [28,29]. The concentration-dependent data indicate that at equivalent concentrations of captopril and a specific inhibitor of NAD(P)H oxidase, diphenylene iodonium, similar inhibition of the production of reactive oxygen species can be observed. *In vivo* the administration of quinapril reduced the PMA-induced ROS generation. The PMA-induced ROS generation in the patients before the quinapril administration was not significantly different to the control subjects. This may indicate that the inhibition of ROS generation *in vivo* by quinapril is only brief acting.

The above-mentioned findings further offer a novel mechanism of certain angiotensin-converting enzyme inhibitors. The direct inhibitory effect of quinaprilat and captopril on NAD(P)H oxidase could be the clue to other effects of angiotensin-converting enzyme inhibitors observed *in vivo* and in cellular assays, as those cellular effects of angiotensin-converting enzyme inhibitors are downstream from NAD(P)H oxidase, which represents one main cellular source of reactive oxygen species. The exact mechanism whereby quinaprilat and captopril inhibit NAD(P)H oxidase has not yet been elucidated. The binding site of quinapril or captopril to NAD(P)H oxidase has not been established, neither has the specific structure been identified, by which quinapril or captopril are enabled to inhibit NAD(P)H oxidase. *In vitro*, quinaprilat and captopril reduced the production of reactive oxygen species, whereas enalaprilat and lisinopril showed no effect. It is tempting to speculate that structural differences among angiotensin-converting enzyme inhibitors might be responsible for these differences. However, the present study does not rule out the possibility that *in vivo* all angiotensin-converting enzyme inhibitors might show a ROS reducing effect.

In summary, the results document that quinapril and captopril inhibit the production of ROS in human cells in concentrations usually reached after therapeutical administration.

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