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Captopril inhibits the oxidative modification of apolipoprotein B-100 caused by myeloperoxydase in a comparative in vitro assay of angiotensin converting enzyme inhibitors

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Abstract

The oxidative modification of low-density lipoproteins (LDL) is a key event in the formation of atheromatous lesions. Indeed, oxidized derivatives accumulate in the vascular wall and promote a local inflammatory process which triggers the progression of the atheromatous plaque. Myeloperoxidase (MPO) has been mentioned as a major contributor to this oxidative process. It takes part in the oxidation both of lipids by chlorination and peroxidation and of apolipoprotein B-100. Based on recent observations with several anti-inflammatory and thiol-containing drugs, the present study was designed to test the hypothesis that anti-hypertensive agents from the angiotensin converting enzyme (ACE) inhibitors group inhibit the oxidative modifications of Apo B-100 caused by MPO. Captopril, ramipril, enalapril, lisinopril and fosinopril were assessed by measuring: their inhibiting effect on the $MPO/H_2O_2/Cl^-$ system, the accumulation of compound II, which reflects the inhibition of the synthesis of HOCl and the LDL oxidation by MPO in presence of several concentrations of ACE inhibitors. Only captopril, a thiol-containing ACE inhibitor, was able to significantly decrease the oxidative modification of LDL in a dose dependent manner and this by scavenging HOCl. This efficient anti-hypertensive drug therefore appears to also protect against the atherosclerotic process by this newly documented mechanism.

Keywords: Myeloperoxidase; LDL; Captopril; ACE inhibitor; Apolipoprotein B-100

1. Introduction

The oxidative modification of low-density lipoproteins (LDL) is a key event in the formation of atheromatous lesions. Indeed, oxidized derivatives accumulate in the vascular wall and promote a local inflammatory process which triggers the progression of the atheromatous plaque (Berliner and Heinecke, 1996; Malle et al., 2000; Ueda et al., 2004). However, the way LDL are oxidized is still unclear as well as the contribution of the sub-endothelial oxidation by monocyte-derived macrophages (Chisolm and Steinberg, 2000; Zouaoui Boudjeltia et al., 2004).

Since a couple of years, myeloperoxidase (MPO) has been mentioned as a major contributor to this oxidative process. Indeed, the enzyme is involved in the production of hypochlorous acid (HOCl), a very reactive oxygen-derived species which takes part in the oxidation both of lipids by chlorination and peroxidation (Podrez et al., 2000) and of apolipoprotein B-100 (Apo B-100) (Carr et al., 2001). Furthermore, MPO is a highly cationic protein (isoelectric point >10) which is able to bind to LDL. Such a phenomenon contributes to the oxidation of these lipoproteins and potentially limits the scavenging by antioxidants (Carr et al., 2000). Nevertheless, various arguments have been put forward

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showing that HOCl produced by the $MPO/H_2O_2/Cl^-$ system mainly acts on the protein moiety of LDL (Winterbourn and Kettle, 2000; Hazell et al., 1994; Yang et al., 1999). The relevance of this observation as a pathogenic factor for atheromatous lesions led some of us to develop an Enzyme-Linked ImmunoSorbent Assay (ELISA) test involving a specific monoclonal antibody for the quantification of the oxidative modification of Apo B-100 (Moguilevsky et al., 2004). It was possible to demonstrate with this procedure that the modification can actually take place at the surface of endothelial cells, therefore constituting an additional mechanism to sub-endothelial oxidation in atheromatous lesions (Zouaoui Boudjeltia et al., 2004).

Based on recent observations with several anti-inflammatory and thiol-containing drugs (Nève et al., 2001; Van Antwerpen et al., 2005), the present study was designed to test the hypothesis that anti-hypertensive agents from the angiotensin converting enzyme (ACE) inhibitors group such as captopril, lisinopril, ramipril, enalapril maleate and sodium fosinopril (see Fig. 1) or of their active counter-parts inhibit the oxidative modification of Apo B-100 caused by MPO and this in spite of a steric hindrance due to the large size of LDL that could mask the active site of the enzyme (Carr et al., 2000). When necessary, the investigation was performed with the derivatives obtained after enzymatic hydrolysis of their prodrugs with a porcine liver esterase (Gana et al., 2002) or by alkaline hydrolysis (Jemal et al., 1985). The inhibition was examined in absence and in presence of LDL using a human recombinant MPO produced by Chinese hamster ovary cell line (Moguilevsky et al., 1991). The procedure included the measurement of the interaction with HClO (Van Antwerpen et al., 2005), the direct interaction with MPO (Nève et al., 2001; Van Antwerpen et al., 2005) and the dose–response relationships between the inhibition of LDL oxidation and several drug concentrations, using an newly developed ELISA assay specific for the quantification of MPO oxidized Apo B-100 (Moguilevsky et al., 2004; Van Antwerpen et al., 2005).

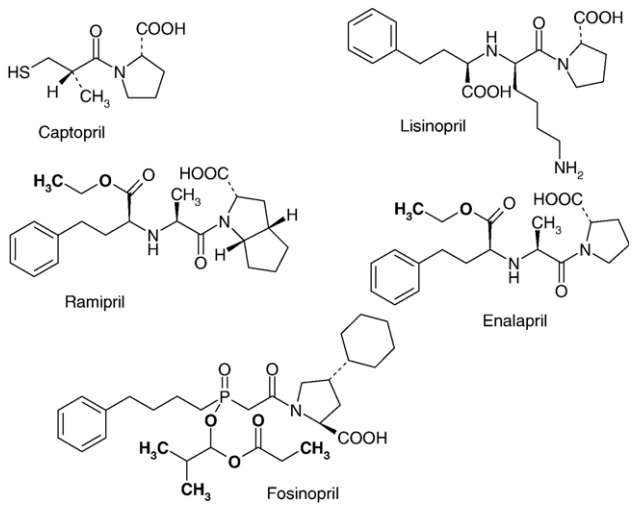

Fig. 1. Structure of the studied ACE inhibitors.

2. Materials and methods

2.1. Chemicals

ACE inhibitors were kindly provided by SMB-Galephar (Belgium). Hydrogen peroxide (H_2O_2), KI, HCl, $EDTANa_2H_2$, $MgCl_2{\cdot}6H_2O$, $NaHCO_3$, H_2SO_4, diethanolamine, polysorbate 80 and NaOH were from VWR (Leuven, Belgium). Bovine serum albumine (BSA), NaN_3, paranitrophenyl phosphate, tris(hydroxymethyl)aminomethane (TRIS), captopril, methionine and catalase were purchased from Sigma (St. Louis, USA). Ammonium acetate, KOH, KH_2PO_4 (VWR International, Leuven, Belgium), methanol and acetonitrile (Fischer Scientific, Loughborough, UK) were LC analytical-grade reagents, used to prepare the mobile phase of the LC system. A PBS buffer (pH 7.4) was prepared at a final concentration of 10 mM of phosphate ions (KH_2PO_4/KOH) and 150 mM of NaCl (all from VWR, Leuven, Belgium). The same phosphate buffer (pH 7.4) was also prepared without NaCl. For LDL preparation and oxidation, a PBS buffer at pH 7.2 was prepared at a final concentration of 2.8 mM of $EDTANa_2H_2$. A pH 7.5 TRIS saline buffer (TBS 80) containing 50 mM of TRIS, 300 mM of NaCl and 0.1% of polysorbate 80 was used for the ELISA assay. Finally, a pH 9.8 diethanolamine buffer was extemporarily prepared by dissolving 0.101 g of $MgCl_2{\cdot}6H_2O$ and 0.2 g of NaN_3 in water with 97 ml of diethanolamine. The pH was adjusted to 9.8 with HCl and the solution was diluted to 1000.0 ml. A stock solution of NaOCl (13% w/w, Aldrich, Steimheim, Germany) was prepared and kept at 4 °C. Before use, a working solution was prepared by dilution (80 μl/50 ml water) and HOCl concentration was determined at pH 6.2 by iodometry with a 20 mM KI solution. Iodine formed was measured at 350 nm and the concentration determined using an extinction coefficient of 22900 M^{-1} cm^{-1} (Gressier et al., 1995). De-oxygenated milliQ water was used for the preparation of all solutions.

2.2. Hydrolysis of the prodrugs

Ramiprilat and enalaprilat were obtained by hydrolysis of their esters with a porcine liver esterase (Gana et al., 2002). Four millimolars of these derivatives were dissolved in 50.0 ml of a pH 8.6 buffer solution with KH_2PO_4 (20 mM) containing 50 U of esterase and the mixture was incubated at 37 °C. Fosinoprilat was obtained by a simple alkaline hydrolysis of the sodium salt at room temperature (Jemal et al., 1985) by dissolving the drug in 50.0 ml of a KOH 0.1 M solution. The hydrolysis was followed by liquid chromatography as described below. After completion of the reaction, 25.0 ml of each mixture was taken up and adjusted to pH 7.4 by H_2SO_4 and the solution was adjusted to 50.0 ml with milliQ water.

2.3. Liquid chromatographic system

Liquid chromatographic systems were developed to both follow the hydrolysis of prodrugs and quantify the remaining quantity of molecules after the reaction with the $MPO/H_2O_2/Cl^-$ system. The hydrolysis of the prodrugs was followed by a liquid chromatography (Waters, Milford, USA) with a mobile phase consisted in a

mixture of a phosphoric acid solution in water (0.2%) and methanol. The proportions of the two solvents and the flow rate were adjusted to obtain the most satisfactory separations: ramipril, 30/70 and 1 ml/min; enalapril, 65/35 and 1 ml/min; fosinopril, 28/72 and 1.5 ml/min. The column was a Symmetry C18 15 cm×4.6 mm, 5 μm with a guard column, 5×4.6 mm, 5 μm (Waters, Milford, USA) and the detection was performed at 254 nm by monitoring the U–V absorbance with a Waters (Milford, USA) diode array detector. The interaction of the active compounds (ramiprilat, enalaprilat and fosinoprilat) with the MPO/H_2O_2/Cl^- system was assessed by the same liquid chromatography conditions.

However, the interaction of the prodrugs (ramipril, enalapril and fosinopril) and lisinopril with the MPO/H_2O_2/Cl^- system was investigated by the same liquid chromatography system where the mobile phase consisted in a mixture of pH 8.0 ammonium acetate solution (0.2%) and acetonitrile. The proportions of the two solvents had to be varied between 100/0 and 50/50 for the different studied drugs in order to obtain the most satisfactory separations at a flow rate of 1 ml/min.

2.4. Preparation of the recombinant enzyme and isolation of LDL

Recombinant MPO was prepared as previously described (Moguilevsky et al., 1991). Each batch solution was characterized by its protein content (mg/ml), its activity (U/ml) and its specific activity (U/mg). The chlorination activity was determined according to Hewson and Hager (1979). Human plasma served for the isolation of LDL by ultracentrifugation according to Havel et al. (1955). Before oxidation, the LDL fraction ($1.019 < d < 1.067$ g/ml) was desalted by two consecutive elutions through PD10 gel-filtration columns (Amersham Biosciences, The Netherlands) using PBS buffer. The different steps were carried out in the dark and the protein content was measured by the Lowry assay for both MPO and LDL (Peterson, 1977). Two batches of MPO were used in the experiments, the first (0.47 mg/ml, 25 U/ml, 53 U/mg) for investigation of the inhibition of the MPO/H_2O_2/Cl^- system and of the oxidation of LDL and the second (0.644 mg/ml, 34 U/ml, 53 U/mg) for the measurement of accumulation of compound II.

2.5. Experimental models

2.5.1. Inhibition of the MPO/H_2O_2/Cl^- system

The inhibition of the MPO/H_2O_2/Cl^- system by captopril was assessed as previously described (Van Antwerpen et al., 2005). In a final volume of 1 ml, the reaction mixture contained the following reagents at the final concentrations indicated between brackets: PBS buffer pH 7.4 (10 mM/140 mM), the drug (600 μM), a gradient of methionine (0 to 600 μM), MPO (~51 nM) in order to produce 60 μM of HOCl during 5 min of incubation at 37 °C with H_2O_2 (100 μM). The reaction was stopped by the addition of 100 μl of catalase (4 U/μl). The remaining quantity of captopril was detected according to Vanderbist et al. (1996) by addition of 1000 μl of a solution of DTNB (4 mM) and 4000 μl of water and measurement of the absorbance at 412 nm. The remaining quantity of the other ACE inhibitors was investigated using the liquid chromatographic system described above. The results were expressed as the remaining quantity of drugs (in %) as a function of methionine concentration (mean±S.D. for $n=5$).

2.5.2. Accumulation of compound II

The interaction of the drugs with the native enzyme and its different oxidized forms was assessed by measuring compound II lifetime in two different conditions consisting in the presence or the absence of Cl^-. The method was adapted from Nève et al. (2001). In a 1.5 ml quartz cell, the following reagents were introduced, at a final concentration stated between brackets, for a final volume of 1.0 ml: 100 μl of MPO (~767 nM), 400 μl of pH 7.4 KH_2PO_4/KOH phosphate buffer (10 mM) with or without NaCl (300 or 0 mM), 250 μl of a drug solution (500 μM) and 50 μl of water. The reaction was initiated by addition of 100 μl of H_2O_2 (30 μM) and absorbances were simultaneously monitored with a diode-array spectrophotometer (Agilent 8453, Palo Alto, CA, USA) at wavelengths characteristic of compound II (456 nm) and of the native enzyme (430 nm). Compound II lifetime was measured as the time for intersection of absorbance curves at 430 and 456 nm in the presence of a drug inducing an inhibition of MPO chlorinating activity. This time corresponds to the complete conversion of compound II to native enzyme, which is demonstrated by a shift in the enzyme spectrum. The results were expressed as mean±S.D. of 3 independent values and compared by an analysis of variance (ANOVA) test.

2.5.3. Inhibition of LDL oxidation

The oxidation of LDL was carried out at 37 °C in a final volume of 500 μl. The reaction mixture contained the following reagents at the final concentrations indicated between brackets: pH 7.2 PBS buffer, MPO (1 μg/ml), LDL (1000 μg/ml), 2 μl of HCl 1N (4 mM), one of the drugs at varying concentrations (5, 15, 30, 300 μM) and H_2O_2 (100 μM). The reaction was stopped after 5 min by cooling the tubes in ice.

The specific measurement of LDL oxidation by the MPO/H_2O_2/Cl^- system was performed by a recently developed ELISA assay based on the binding between a mouse monoclonal antibody recognizing the oxidized LDL and an anti-mouse immunoglobulin G (Ig G) coupled with alkaline phosphatase. The amount of oxidized LDL was quantified by measurement of the phosphatase activity and expressed as the absorbance value of the reaction product of the alkaline phosphatase, i.e. *para*-nitrophenol (405 nm). The assay was performed as described by Moguilevsky et al. (2004) in a NUNC maxisorp plate (VWR, Zaventem, Belgium): 200 ng/well of LDL were coated overnight at 4 °C in a sodium bicarbonate pH 9.8 buffer (100 μl). Afterwards, the plate was washed with TBS 80 buffer and then saturated during 1 h at 37 °C with the PBS buffer containing 1% of BSA (150 μl/well). After washing the wells twice with the TBS 80 buffer, the monoclonal antibody Mab AG9 (200 ng/well) obtained according to a standard protocol (Moguilevsky et al., 2004) was added as a diluted solution in PBS buffer with 0.5% of BSA and 0.1% of polysorbate 20. After incubation during 1 h at 37 °C, the plate was washed 4 times with the TBS 80 buffer and a 3000 times diluted solution of Ig G anti-mouse alkaline phosphatase (Promega, Leiden, The Netherlands) in the same buffer was added (100 μl/well). The wells were washed again

Table 1
Determination of the extent of inhibition of the $MPO/H_2O_2/Cl^-$ system by the different drugs investigated

Drugs	IC_{50} (μM)	taccII with Cl^- (s)	taccII without Cl^- (s)
Blank	–	ND	1282±19
Captopril	58±1	ND	497±22
Lisinopril	<1	ND	1142±117
Ramipril	<1	ND	1254±51
Ramiprilat	<1	ND	1298±19
Enalapril maleate	<1	ND	1273±38
Enalaprilat	<1	ND	1281±50
Fosinopril sodium	–	ND	1258±20
Fosinoprilat	–	ND	1266±35

Results are expressed as the quantity of methionine required to inhibit 50% of the activity of the system (IC_{50}) and accumulation of compound II is assessed by the measurement of the evolution of absorbance at wavelengths typical to the native enzyme (430 nm) and to compound II (456 nm) as a function of time (taccII).
ND: compound II not detected; results are expressed as mean±S.D. for $n=3$.

4 times and a revelation solution (150 μl/well) containing 5 mg of paranitrophenyl phosphate in 5 ml of diethanolamine buffer was added during 30 min at room temperature. The reaction was stopped with 60 μl/well of NaOH 3 N solution. The measurement of the absorbance was performed at 405 nm with a background correction at 655 nm using a Bio-Rad photometer for 96 wells plate (Bio-Rad laboratories, CA, USA). Results were expressed as the mean±S.D. of the percentage of LDL oxidation for 6 independent measurements.

3. Results

3.1. Inhibition of the $MPO/H_2O_2/Cl^-$ system

In the experimental model used, a drug which is efficiently oxidized by the MPO system causes an increase in the quantity of methionine required to inhibit this oxidation. As a consequence, a potent inhibitor of the MPO system will have a relatively high IC_{50} value, which corresponds to the concentration of methionine required to inhibit 50% of its oxidation by the MPO system (Van Antwerpen et al., 2005). Considering the potential inhibition of the $MPO/H_2O_2/Cl^-$ system by the different studied molecules, Table 1 shows that only captopril had a statistically significant inhibiting effect ($P<0.01$). Indeed, 58±1 μM of methionine were necessary to inhibit 50% of captopril oxidation. This quantity decreased to values less than 1 μM with the other compounds and no inhibiting effect was observed with fosinopril and fosinoprilat.

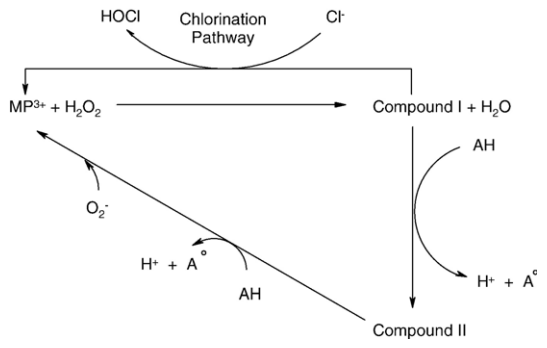

Fig. 2. Representation of MPO redox transformation catalytic pathways. MP^{3+}: native enzyme; compound I: MP^{3+} H_2O_2; compound II: MP^{2+} H_2O_2; AH: reducing agent; O_2^-: superoxide anion. Adapted from Kettle and Winterbourn (1988).

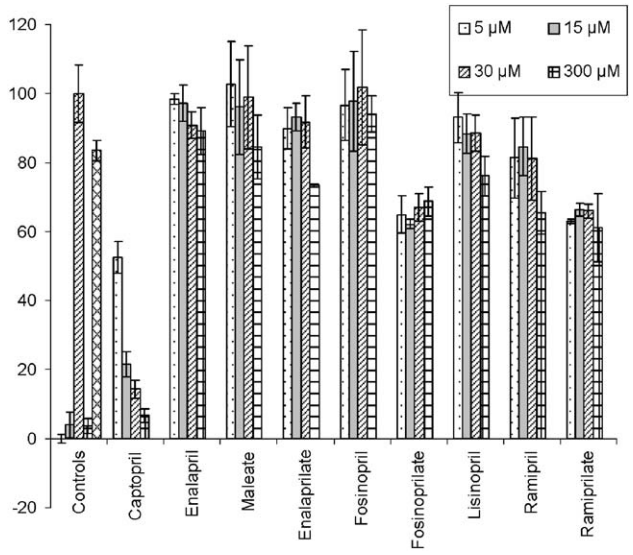

Fig. 3. LDL oxidation (in %) in relation to different concentrations of thiol-containing molecules and flufenamic acid: 5 μM, 15 μM, 30 μM and 300 μM. Absorbance of LDL (Blank; controls, □) and oxidized LDL without drugs (controls, ▨) are respectively considered equal to the 0 and 100%. In absence of H_2O_2, %=8.5±0.9% (controls, ■); in presence of catalase (400 U/ml) (controls, ▦), %=7±1%; in presence of esterase (1 U) (controls, ▩), %=84±3%. Results are the mean±SD for $n=6$.

3.2. Accumulation of compound II

Fig. 2 shows that MPO is oxidized by H_2O_2 to give compound I, which is able to produce HOCl in the presence of chloride ions (Cl^-) by returning to the native enzyme state. However, in conditions such as the absence of Cl^-, the presence of an excess of H_2O_2 or the presence of a drug able to inhibit the formation of HOCl, the system evolved to the promotion and the accumulation of compound II, a reduced form of compound I (Arnhold et al., 2003; Furtmüller et al., 2000; Marquez et al., 1994). Assuming these statements, the measurements of compound II lifetime were carried out with and without chloride anions. Values reported in Table 1 show that no accumulation of compound II could be observed in presence of chloride anions, which demonstrates the absence of inhibition of HOCl synthesis by the investigated molecules (Van Antwerpen et al., 2005; Nève et al., 2001). However, in absence of chloride anions, only captopril was able to interact directly with compound II (ANOVA test, $P<0.01$).

3.3. Inhibition of LDL oxidation

The measurement of the LDL oxidation focused on the oxidative modification of the Apo B-100 by the $MPO/H_2O_2/Cl^-$ system. The oxidized protein was recognized by a specific monoclonal antibody and used in the ELISA test. Considering that native LDL is the blank and that the measurement without the

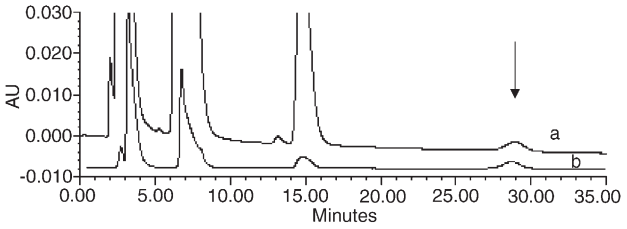

Fig. 4. Chromatograms of the reaction of enalaprilat with the $MPO/H_2O_2/Cl^-$ system and HOCl in the following conditions; a: pH 7.4 PBS buffer (NaCl 300 mM and PO_4^{3-} 10 mM), enalaprilat (600 μM), MPO (51 nM), H_2O_2 (100 μM); b: pH 7.4 PBS buffer, enalaprilat (60 μM) and HOCl (60 μM). The peak marked with ↓ corresponds to the product of oxidation by the both systems.

drug constitutes 100% of oxidation, Fig. 3 shows the percentage of LDL oxidation related to several drug concentrations. As a matter of fact, only captopril significantly inhibited LDL oxidation in a dose dependent manner (5 μM: 53±5%, 15 μM: 22±4%, 30 μM: 14±3%, 300 μM, 7±2%, $P<0.01$). Most of other ACE inhibitors did not show any observable effect.

4. Discussion

The study of the interaction of drugs with the $MPO/H_2O_2/Cl^-$ system is essential to assess the possibility they can inhibit LDL oxidation. The inhibition of the MPO chlorinating activity can be easily measured by assessing the chlorination of taurine (Nève et al., 2001) or of monochlorodimedone (Jerlich et al., 2000) in the presence of molecules under investigation. However, both thiol-containing derivatives (captopril) and chloramines, potential products of the reaction between HOCl and amines (e.g. ramipril), interfere with such systems (data not shown). In order to be able to compare the different studied molecules in a system involving a chemical competition towards the $MPO/H_2O_2/Cl^-$ system, some of us proposed to use methionine as a competitor and developed an original procedure to this purpose (Van Antwerpen et al., 2005). In these conditions, a molecule which reacts with the $MPO/H_2O_2/Cl^-$ system, requires a higher amount of methionine to inhibit its oxidation. The experimental data showed that captopril was more efficient than the other ACE inhibitors probably as the consequence of the presence in the molecule of a thiol function that has already been described as highly reactive towards HOCl ($>10^7$ M^{-1} s^{-1}; Folkes et al., 1995; Peskin and Winterbourn, 2001) and is able to compete with methionine (bearing a methylsulfide function). The other ACE inhibitors (excepted fosinopril) have an amine function which has a kinetic rate constant of about 10^5 M^{-1} s^{-1} (Folkes et al., 1995) and does not react with HOCl in presence of methionine. Furthermore, fosinopril and fosinoprilat do not contain chemical functions that could interact with HOCl, such as supported by the absence of interaction with the $MPO/H_2O_2/Cl^-$ system.

The results therefore suggest that the reaction of these drugs is essentially due to a single chemical reaction with HOCl without any interaction with the enzymatic system. This statement is corroborated by the results of the liquid chromatographic assay in which the occurrence of a similar identical peak could be detected for ramipril, ramiprilat, lisinopril, enalapril and enalaprilat in presence both of HOCl and of the $MPO/H_2O_2/Cl^-$ system (e.g. enalaprilat, Fig. 4). This suggests that the oxidation pathway is similar for these derivatives and is due to the reaction with HOCl. Moreover, the results could be also related to the data obtained concerning the accumulation of compound II: the observation that this form could not be detected in the presence of chloride anions demonstrated the absence of antagonism between the studied molecules and chloride anions in the synthesis of HOCl such as precisely observed by us with flufenamic acid or with several oxicams (Nève et al., 2001; Van Antwerpen et al., 2005). As the accumulation of compound II reflects the ability of a particular compound to inhibit the synthesis of HOCl, our observations indicate that these molecules can only act by scavenging HOCl. In a similar assay performed in absence of chloride anions, captopril was the sole molecule able to reduce compound II by behaving as an electron donor (see Fig. 1). If the thiol function is a key parameter in this mechanism, other authors including our group have also demonstrated that the size of the molecule could play an important role (Burner et al., 1999; Van Antwerpen et al., 2005). As a matter of fact, other ACE inhibitors that were unable to scavenge compound II have a larger size that is unfavourable to this purpose.

Concerning the inhibition of the LDL oxidation, the results resumed in Fig. 3 perfectly corroborated those described above. Indeed, captopril which efficiently scavenged HOCl in the first experiment, was able to reduce the amount of oxidized LDL in spite of the binding between MPO and LDL. On the contrary, the other ACE inhibitors were unable to inhibit LDL oxidation, probably as a consequence of their inability to inhibit MPO or to efficiently scavenge HOCl. At physiological concentrations (Jankowski et al., 1995), the thiol-containing ACE inhibitor clearly exerted a protective effect during the oxidative modification of LDL by the $MPO/H_2O_2/Cl^-$ system. Such a protection of LDL from oxidation, clearly demonstrated by the present study, has already been suggested by several authors in studies in animals or in in vitro models. De Nigris et al. (2001) demonstrated that a chronic treatment with captopril reduced the susceptibility of plasma LDL to in vitro oxidation, leading to the decrease of the atherosclerotic lesion in Apo E knockout mice. They correlated these results showing a significant inhibition of the MDA production in copper induced LDL oxidation. On the other hand, Fernandes et al. (1996) showed that captopril was able to significantly reduce lipid peroxidation and oxidation of LDL by copper ions. In conclusion, captopril which is an efficient anti-hypertensive drug inhibiting the angiotensin converting enzyme, could also protect against the atherosclerotic process by inhibition of the oxidative modification of Apo B-100 in LDL. However, some clinical data are necessary to assess the contribution of this new mechanism in the anti-atherosclerotic effect of captopril.

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