Biochemical Pharmacology, Vol. 40, No. 9, pp. 2169–2175, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

PROTECTIVE EFFECTS OF SULFHYDRYL-CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORS AGAINST FREE RADICAL INJURY IN ENDOTHELIAL CELLS

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(*Received* 2 *March* 1990; *accepted* 5 *June* 1990)

Abstract—The effects of SH-containing (captopril, epi-captopril, and the free-SH form of zofenopril) and non-SH-containing (enalaprilat and lisinopril) angiotension converting enzyme (ACE) inhibitors on free radical injury in cultured endothelial cells were studied. When cultured endothelial cells were exposed to a superoxide and hydroxyl radical generating system (dihydroxyfumarate + Fe^{3+}-ADP) for 30 min, lipid peroxidation [malondialdehyde (MDA) formation] occurred, and cellular viability (trypan blue exclusion) decreased to 41%; concomitantly, plasma membrane blebbing, assessed by scanning electron microscopy, occurred in 65% of the cells. Preincubation of the cells with each of the SH-agents before free radical addition resulted in an equipotent concentration-dependent (10–200 μM) inhibition (15–60%) of MDA formation; both losses in cellular viability and percent blebbed cells were reduced significantly ($P < 0.05$) by concentrations as low as 10 μM of each SH-agent. However, neither of the non-SH agents up to 200 μM produced any major effect. When the effects on hydroxyl radical formation in the system were assessed by ESR spin-trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), concentrations of 10 and 50 μM of the SH-agents reduced the intensity of the DMPO-OH adducts 20 and 50% respectively. Similar results were observed when the hydroxyl radical was generated from the Fenton-reagents ($Fe^{2+} + H_2O_2$), suggesting direct hydroxyl radical scavenging. Thus, these results demonstrate that the SH-containing ACE agents are capable of protecting the endothelial cells against free radical induced lipid peroxidation and cell injury; the mechanism may be due to direct hydroxyl radical scavenging.

The efficacy of captopril and other angiotensin converting enzyme (ACE) inhibitors in the treatment of hypertension and congestive heart failure has been well documented and ascribed primarily to their ACE inhibitory actions [1, 2]. However, captopril was also found to be capable of providing protection against ischemia/reperfusion-induced arrhythmias, a property not shared by other ACE inhibitors [3]. These findings suggested that these beneficial effects might not be dependent on angiotensin inhibition alone. Recently, experimental studies by Westlin and Mullane [4] demonstrated that captopril improves post-ischemic contractile derangements by a mechanism independent of ACE inhibition; their studies suggested that the effect could be due to free radical scavenging ability of the sulfhydryl (SH) moiety of the agent. Since free radical damage of vascular etiology could be an important mechanism of myocardial ischemia-reperfusion injury [5], we used cultured endothelial cells to study the effects of both SH- and non-SH-containing ACE inhibitors on free radical-mediated lipid peroxidation, plasma membrane blebbing, and cell death. In association, their relative free radical scavenging effects were investigated by the technique of electron spin resonance (ESR) spin trapping using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as the spin trap.

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MATERIALS AND METHODS

Captopril, its stereoisomer, epi-captopril, and the arginine salt of zofenopril were obtained from the Squibb Medical Institute (Princeton, NJ). Enalaprilat and lisinopril were provided by Merck Sharp & Dohme Research Laboratories (Rahway, NJ). Dihydroxyfumarate (DHF), $FeCl_3 \cdot 6H_2O$, ADP, and 2-thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO). DMPO was purchased from Aldrich Chemical (Milwaukee, WI) and was purified by activated charcoal before each experiment.

Bovine pulmonary arterial endothelial cells were obtained from the American Tissue Culture Co. (Rockville, MD; ATCC No. CCL 207) and cultured in Medium 199 supplemented with penicillin (50 units/mL), streptomycin (50 μg/mL), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, 10 mM), and GIBCO fetal calf serum (20%). Confluent plates were trypsinized by 0.05% trypsin in 0.02% EDTA solution (Sigma). The digestion was stopped by adding 5 mL of growth medium with serum. The cells were pelleted and washed twice with the incubation buffer at 37° and resuspended in the same buffer at 7–10 million cells/mL.

Free radicals were generated by a superoxide-producing (from 1.67 mM DHF) and iron-catalyzed (50 μM $FeCl_3$ chelated by 500 μM ADP) system as described previously [6, 7]. Endothelial cells (0.8–

1×10^6/mL) were resuspended in the incubation buffer containing 5 mM glucose, 5 mM sodium-acetate, 5 mM sodium-pyruvate, 135 mM NaCl, 1.2 mM $MgCl_2$ and 10 mM potassium phosphate, pH 7.2. The selected ACE inhibitors (10–200 μM) were preincubated for 5–10 min before the final addition of DHF and Fe^{3+}-ADP. All samples were incubated for 30 min at 37°. Cellular lipid peroxidation was measured by TBA reactant formation which was expressed as malondialdehyde (MDA) equivalents/mg protein [6, 7].

Cellular viability was determined morphologically by trypan blue (0.1%) exclusion. Plasma membrane blebbing was monitored by scanning electron microscopy (SEM). The samples after 30 min of incubation were fixed overnight in 3% glutaraldehyde, in a 0.1 M phosphate buffer at pH 7.4. Subsequently, aliquots of the cell suspension were placed onto polylysine coated glass cover slips for 45 min. The attached cells were then dehydrated through a graded series of ethanol, and critically point dried using CO_2 as the exchange fluid. Finally, the cover slips were mounted on SEM stubs and sputter coated with a conducting material-gold, and visualized in a JEOL scanning electron microscope (model JSM 35). A minimum of 500 cells were randomly analyzed for each sample.

Hydroxyl radical production, using DMPO (45 mM) as the spin trap, was monitored by ESR spectroscopy as described [8, 9]. The effects of each of the ACE inhibitors on the relative hydroxyl radical concentrations generated by the DHF + Fe^{3+}-ADP system or by $FeSO_4$ (50 μM) + H_2O_2 (2 mM) were assessed by the relative signal intensity of the DMPO-OH adduct [9]. All ESR measurements were determined at room temperature with a Bruker ER-100 X-band spectrometer using 100 kHz field modulation. Instrumental conditions were: power, 10 mW; modulation amplitude, 1.25 G; time constant, 0.5 sec; scan time, 200 sec; scan range, 100 G and gain, 1.25×10^5.

All values are means ± SD of three to six separate determinations. Statistical analyses were performed by unpaired Student's *t*-test.

RESULTS

Effects on cellular lipid peroxidation. We have utilized DHF + Fe^{3+}-ADP as a convenient oxy-radical system to study peroxidative injury in various subcellular membranes and organelles [6–10]. In the present study, when the cultured endothelial cells were incubated with the free radical system for 30 min, cellular lipid peroxidation occurred as indicated by more than a 4-fold increase in TBA-reactive product formation compared with cells incubated in the buffer alone (11.7 ± 1.2 vs 2.7 ± 0.7 nmol MDA equivalents/mg protein, $P < 0.001$). With the addition of captopril before the free radical components, MDA formation in the cells was inhibited progressively (15–60%), by increasing levels (10–200 μM) of captopril (Fig. 1). In an effort to further clarify the nature of the captopril-mediated anti-peroxidative effect, several other ACE inhibitors with different ACE inhibiting activities and with and without the free SH group were examined. In Fig. 2, the relative anti-peroxidative potencies of captopril, epi-captopril (a stereoisomer of captopril), and zofenopril* (an arginine blend of zofenopril containing a free SH group) were compared. Epi-captopril is 100-fold less potent than captopril as an ACE inhibitor, whereas zofenopril is 5-fold more potent than captopril [11]. The data indicate that all three SH-agents exhibited similar concentration-related anti-peroxidative activities: 50 μM and 200 μM concentrations of each agent inhibited about 28 and 60% ($P < 0.05$ or less), respectively, of cellular MDA formation. For comparison, both enalaprilat and lisinopril (up to 200 μM) did not provide significant protection against lipid peroxidation.

Protective effects on cellular viability and blebbing. The protective effects of the SH-agents against free

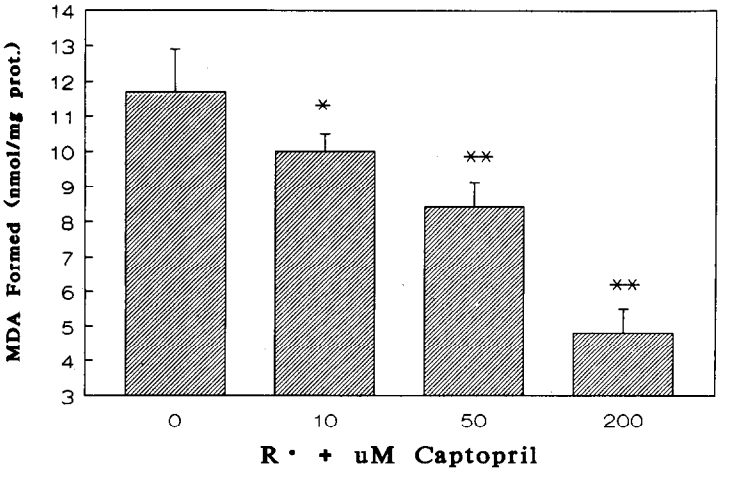

Fig. 1. Inhibitory effect of captopril on free radical-mediated lipid peroxidation in endothelial cells. The cells were preincubated in buffer with captopril at different levels for 5 min, 37°, before the addition of DHF + Fe^{3+}-ADP (R·). After 30 min of incubation, samples were assayed for total MDA formation. Key: *$P \leq 0.05$, and **$P \leq 0.01$ vs R· control.

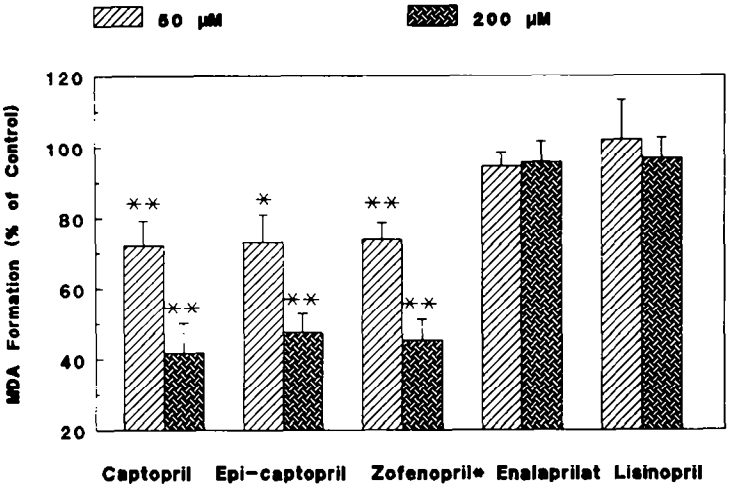

Fig. 2. Comparative inhibitory effects of captopril, epi-captopril, zofenopril*, enalaprilat and lisinopril on endothelial cellular lipid peroxidation. The cells were preincubated without (control) or with each agent at either 50 or 200 μM for 5 min before the addition of DHF + Fe^{3+}-ADP (R·). After 30 min of incubation, samples were assayed for total MDA formation, and the values are expressed as percent of R· controls. MDA formation in the R· controls (100%) = 11.7 ± 1.2 nmol MDA equivalents/mg protein. Zofenopril* = an arginine blend of zofenopril containing a free SH group. Key: *P ≤ 0.05, and **P ≤ 0.01 vs R· control.

radical-induced losses of viability were examined. Incubation of the endothelial cells with the free radical system for 30 min resulted in a 60% loss in viability measured by the trypan blue exclusion method (Fig. 3). For comparison, the control samples incubated with buffer alone for the same period of time maintained 87% viability. Therefore, the net loss of viability due to free radicals was 46%. Captopril as low as 10 μM provided significant protection against the loss of viability (62.5 ± 8 vs 40.6 ± 5% viability, $P < 0.01$), which was close to 50% effective in retarding the induced viability loss (22 vs 46% due to R·). At higher concentrations of captopril (25–100 μM) major reversals of the induced viability losses were observed and at 100 μM almost complete protection was achieved: 82.5 ± 5.6 vs 87 ± 6% (control) viable (P = NS). The relative effectiveness of the three SH-containing ACE agents in preventing cell death was compared. Data in Fig. 3 demonstrated that both epi-captopril and zofenopril* exhibited concentration-dependent protective effects which were remarkably similar to that of captopril.

Cell surface "blebbing" is considered a mani-

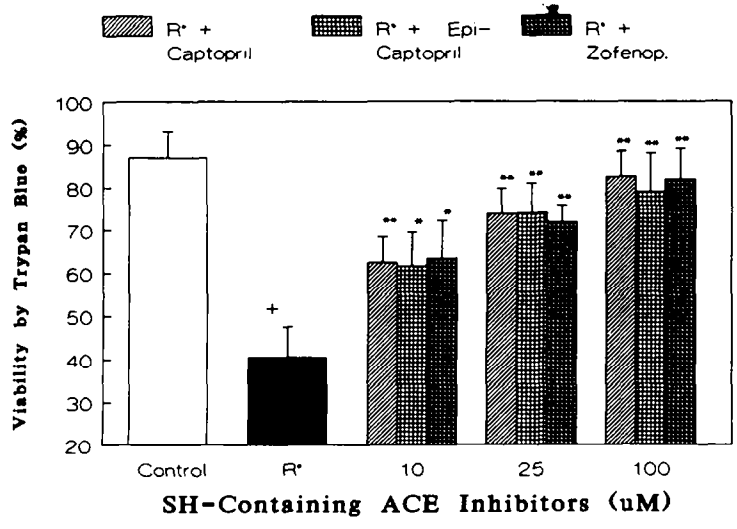

Fig. 3. Comparable protective effects of captopril, epi-captopril and zofenopril* against free radical-mediated loss of endothelial cellular viability. The incubation conditions were as described under Fig. 2, and the cellular viability was determined by trypan blue (0.1%) exclusion. Key: †P ≤ 0.01 vs buffer control; *P ≤ 0.05, and **P ≤ 0.01 vs R· alone.

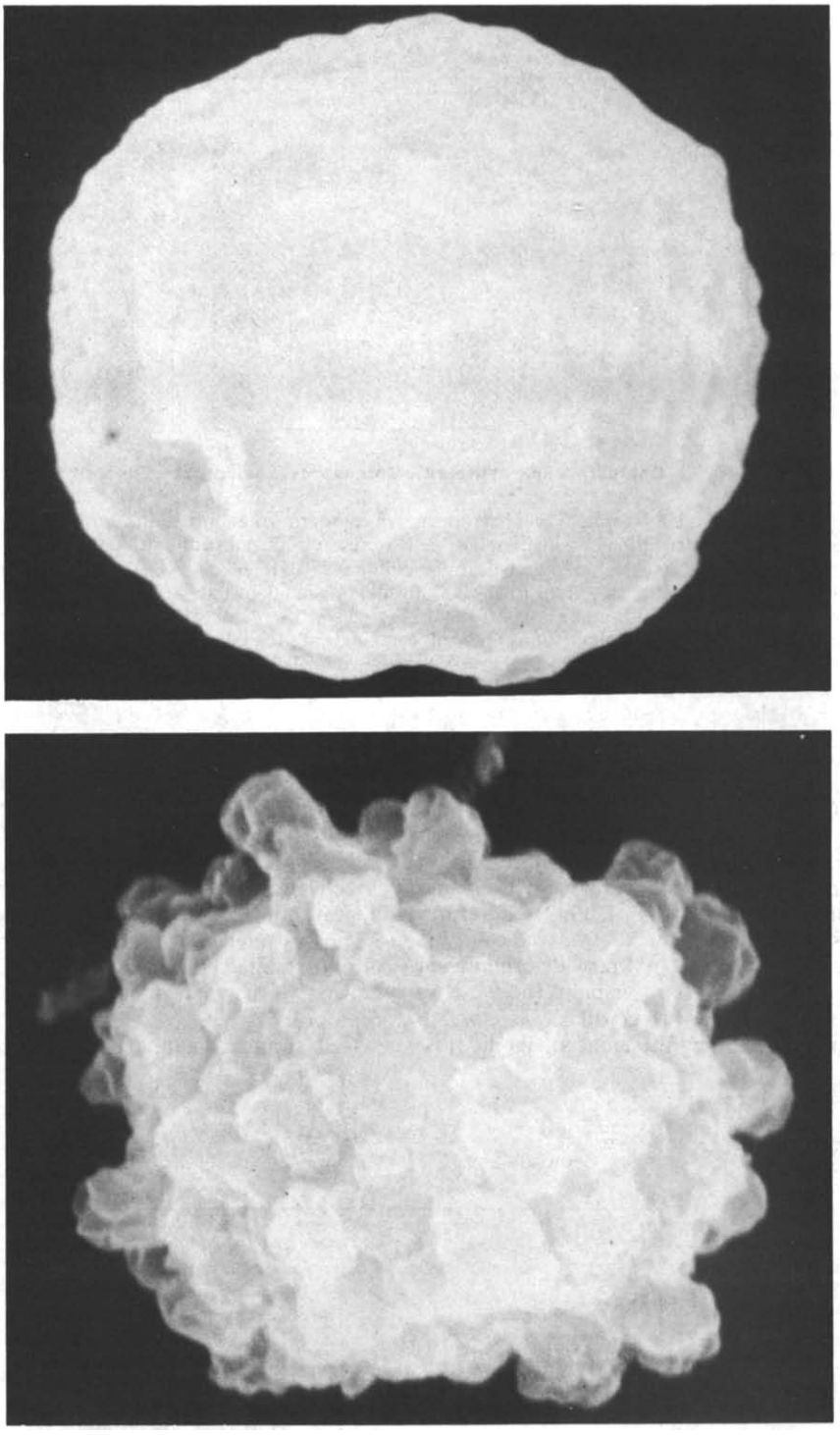

Fig. 4. Scanning electron micrographs of typical isolated endothelial cells incubated at 30° in the absence (top panel) or presence (bottom panel) of DHF + Fe^{3+}-ADP; at 30 min, 65% of the cells were shown covered with blebs. Magnification: ×13,500.

festation of cytoskeletal alteration resulting from toxic or hypoxic stress to isolated cells [12, 13]. When the endothelial cells were exposed to the free radicals for 30 min, about 65% of originally viable cells exhibited extensive blebbing [Figs. 4 (bottom panel) and 5]; for comparison, only ~10% of the cells incubated in the buffer alone showed blebbing, and the majority of them maintained an intact appearance (Fig. 4, top panel). When the effects of the three SH-containing agents on the blebbing phenomenon

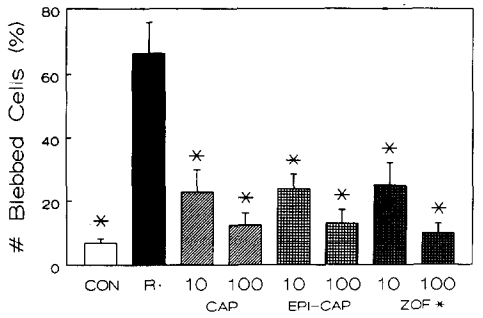

Fig. 5. Protective effects of captopril, epi-captopril and zofenopril* against free radical-mediated endothelial bleb formation. The incubation conditions were as described under Fig. 3. Bleb scoring of at least 500 fixed cells per sample was performed. Key: *P < 0.01 vs R· in the absence of any drug.

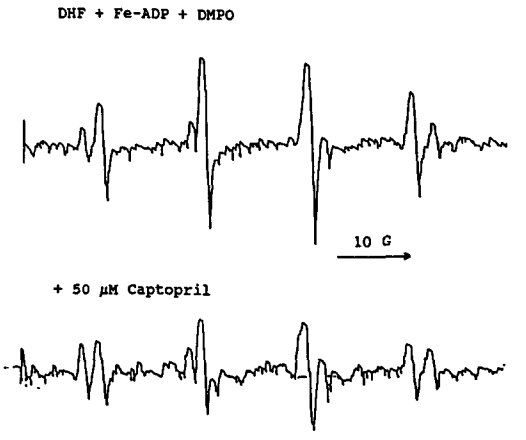

Fig. 6. ESR spectra of DMPO-OH adduct formation in an incubation mixture containing DHF + Fe^{3+}-ADP and DMPO (control), and in the same mixture plus 50 μM captopril. Instrumental conditions: power, 10 mW; modulation amplitude, 1.25 G; time constant, 0.5 sec; scan time, 200 sec; scan range, 100 G; and gain, 1.25×10^5.

were examined, the percentage of blebbed cells was reduced dramatically to about 25% by 10 μM and to 15% by 100 μM of each SH-agent (Fig. 5).

Hydroxyl radical scavenging assessed by ESR. In solution, DHF auto-oxidizes to generate superoxide anions, which in the presence of Fe^{3+}-ADP lead to the formation of hydroxyl radicals. The hydroxyl radical is well-known to be extremely reactive and may mediate both cellular lipid peroxidation and cell death. The generation of hydroxyl radicals in our system has been confirmed by ESR spin-trapping [8, 9]. In the present study, the influence of the SH-agents on the level of hydroxyl radical generated was assessed by ESR using DMPO as the spin trap. As shown in Fig. 6, the production of ·OH was monitored by the DMPO-OH adduct formation which is characterized by the major signal consisting of a 1:2:2:1 quartet with hyperfine coupling constant $a^N = a^H_\beta = 14.9$ G. In the presence of 50 μM captopril, the relative DMPO-OH signal intensity was reduced 50% (Fig. 6). In separate samples, 10 μM captopril reduced the signal by 20%. Similar concentration-related inhibition of the DMPO-OH adduct formation was observed for epi-captopril and zofenopril*. These findings indicated that all three

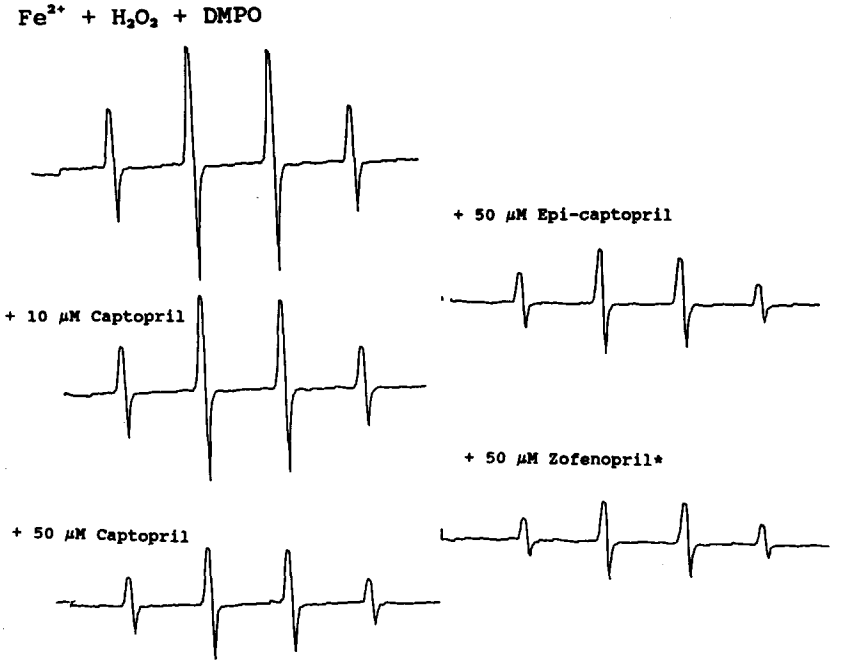

Fig. 7. ESR spectra of DMPO-OH adduct formation in an incubation mixture containing Fe^{2+}/H_2O_2 and DMPO without (control) and with addition of different ACE inhibitors. Experimental and instrumental conditions were as described under Materials and Methods and Fig. 6.

SH-containing ACE inhibitors significantly ($P < 0.05$) reduced the level of hydroxyl radicals generated from the DHF + Fe^{3+}-ADP system. In separate experiments, none of the three SH-agents (up to 200 μM) showed appreciable effects on the rates of DHF auto-oxidation from the free radical system measured by the method of Goscin and Fridovich [14]. In data not shown, neither of the non-SH ACE agents (up to 200 μM) was able to reduce the level of hydroxyl radical as estimated by the relative ESR DMPO-OH intensity.

Since the DHF system initially generates superoxide anions, which then lead to the formation of hydroxyl radicals, the reduction in OH-adduct formation could be due to either superoxide scavenging or direct hydroxyl radical trapping by the SH-containing agents. To further distinguish the two possibilities, the Fenton reagents, consisting of Fe(II) and H_2O_2, were employed to produce hydroxyl radicals. In Fig. 7 the ESR spectrum in the control reveals that DMPO-OH was the only oxygen-centered free radical adduct produced in the system; again, the addition of 10 and 50 μM captopril resulted in reductions of 20 and 50%, respectively, in the relative intensity of the DMPO-OH adduct. Similar effects were observed for the other two SH-containing inhibitors. It appears unlikely that the reduced formation of hydroxyl radicals was due to the SH-agent-mediated reduction of the precursor H_2O_2, which was in millimolar (2 mM) concentration, whereas that of the SH-agents was in the micromolar (10–100 μM) range. These data suggest that the SH-agents are capable of directly scavenging hydroxyl radicals.

DISCUSSION

We have demonstrated that captopril and the related SH-containing ACE inhibitors (at micromolar concentrations) were able to protect the endothelial cells against free radical-mediated membrane blebbing and cell death. In association, all these SH-agents were found to possess relatively potent hydroxyl radical scavenging and moderate anti-peroxidative abilities. The chemical structure of captopril is relatively simple, with the carboxyl group on proline and the carbonyl and thiol groups essential for its activity. It is described that the potency of the agent is enhanced greatly by the sulfhydryl moiety [15, 16]. Zofenopril is a sulfur-containing pro-drug which, after bio-conversion to its active free-sulfhydryl containing form, is five times more potent than captopril [11], whereas epi-captopril, a stereoisomer of captopril, is essentially devoid of any ACE-inhibiting activity. Since our data demonstrate that all three SH-agents were equipotent in inhibiting lipid peroxidation and preventing the induced losses in cellular viability, the protective effects are clearly independent of their ACE inhibition activities. Furthermore, since both non-SH ACE inhibitors were found to be ineffective, the protective effects of the SH-containing agents appear to be related to the presence of the free SH moiety.

Certain sulfhydryl compounds (RSH) are a major class of protective agents against radiation damage [17]. These agents can neutralize free radicals by either a hydrogen atom donating or electron transferring reaction [18]. The mechanism of free radical repair mediated by RSH compounds may involve carbon-centered (e.g. lipid-C·), or oxygen-centered (e.g. LOO·, ·OH) radicals. The endothelial cell death process may involve both membrane lipid peroxidation and protein oxidation mediated by the hydroxyl and related secondary free radicals (such as conjugated oxy-iron species) [19]. It appears that the protective effects of the SH-agents correlate more closely with their direct hydroxyl radical scavenging abilities than with their antiperoxidative potency. However, due to the limitations of the TBA assay [20], the MDA values presented in this study could only be regarded as a semi-quantitative index of cellular lipid peroxidation. It remains to be determined if the lipid antiperoxidative activities of the SH-agents are secondary to the free radical scavenging action(s) in the aqueous phase.

Captopril and the related SH-containing agents are reported to scavenge superoxide anions generated *in vitro*; captopril, but not enalaprilat, is able to alleviate post-ischemic contractile impairment *in vivo* [4]. Since it is believed that myocardial stunning is a reperfusion-induced phenomenon that is mediated at least in part by free radicals [21], it was proposed that captopril-mediated scavenging of superoxide may account for the observed beneficial effect [4]. In our work with myocardial ischemia/reperfusion, using the technique of ESR spin trapping, we have observed the generation of both superoxide and hydroxyl radicals within the first few minutes of reperfusion [22, 23]. In the present study, we used a chemical system to generate both superoxide and hydroxyl radicals for induction of cellular injury. Since the hydroxyl radical is much more reactive than any other oxygen-centered free radical species [17], it may play a major role in the induction of lipid peroxidation and cell death. Our spin-trapping studies indicate that all three SH-containing agents, but not the non-SH-agents, are able to lower the hydroxyl radical level by a direct scavenging action. Presumably, the hydroxyl radical generated in our system could be intercepted by the SH-agents before reaching the cellular sites of injury. Our data suggest that such a reaction mechanism provided the major protective effects on the endothelial cells.

In conclusion, we have demonstrated that the SH-containing ACE inhibitors can provide potent protective effects against oxy-radical mediated endothelial cell injury *in vitro*. However, the clinical relevance of the observations remains to be defined further, as relative high concentrations (10–100 μM) of the SH-containing agents were required to provide protection. For comparison, the upper therapeutic serum level of captopril is up to 5 μM [15]. Nevertheless, concentrations as low as 10 μM of the SH-agents were shown to be able to reverse close to 50% of the induced cell death (Figs. 3 and 5). Oxygen-derived free radicals and their metabolites have been increasingly recognized for their role in tissue injury and cell death during myocardial ischemia and reperfusion [22–25]. In addition, free radical reactions have also been implicated in the pathogenesis of atherosclerosis [26, 27]. According to the response-

to-injury hypothesis proposed by Ross [28], injury to the endothelium is the initiating event in atherogenesis. Due to their constant interaction with blood components, the endothelial cells are potential targets of reactive oxy-radicals released from activated neutrophils (and other cells such as platelets, macrophages) and oxidizable drugs and chemicals. Presumably, the presence of therapeutic agents, such as SH-containing ACE inhibitors which are capable of free radical scavenging, would be of value in providing both ACE inhibition and anti-radical protection to the endothelium.

Acknowledgements—The authors would like to thank Ms. Beth Pflug, Ms. Lisa Kopyta, Ms. Patricia Boehme and Mr. Jin-hu Liang for their excellent technical performance. This work was supported by NIH Grant PO1-HL-380790, R01-HL-36418, the Bristol-Myers Squibb Company, and DOD Grant N0014-88-K-0405.

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