Effects of Captopril on Interleukin-6, Leukotriene $B_4$, and Oxidative Stress Markers in Serum and Inflammatory Exudate of Arthritic Rats: Evidence of Antiinflammatory Activity

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Cytokines play a pivotal role in the initiation, evolution, and persistence of chronic inflammation; and high levels of interleukins (ILs) have been found in synovial fluid (SF) of various arthropathies (Dayer and Fenner, 1992; Bertazzolo et al., 1994). ILs trigger bone resorption (Winrow et al., 1993), intermediate cellular communication in inflamed tissue due to their ability to synergize with other inflammatory mediators (Bertazzolo et al., 1994), and lead to overproduction of free radicals (FRs) by activating leukocytes that infiltrate the inflamed joint (Greenwald, 1990). Eicosanoids are another major class of inflammatory mediators, and with leukotriene $B_4$ (LTB$_4$) being an important member. In a wide variety of inflammatory disorders, LTB$_4$ levels have been elevated and related to the severity of the disease (Crooks et al., 1996; Subeyaz et al., 1996). LTB$_4$ recruits and activates inflammatory cells (Henderson, 1994), stimulates production of cytokines (Ford-Hutchinson, 1990), and thus accentuates FR generation and consequently prolongs tissue inflammation. Consideration has therefore been given to controlling production of specific cytokines and eicosanoids, besides many other mediators, as a therapeutic strategy for patients suffering from inflammatory diseases.

Captopril (CP) is an angiotensin converting enzyme (ACE) inhibitor (Breckenridge, 1988), and, besides its usefulness in hypertension, it provides protection against ischemic/reperfusion (I/R) injury (Birincioglu et al., 1997). Since FRs are implicated in the pathology of I/R injury (Birincioglu et al., 1997) and CP contains a sulphydryl (-SH) moiety, the beneficial effects of CP could possibly lie in a FR scavenging capability. Thus, several investigators have studied this effect for CP; however, seemingly contradictory results have been obtained (Kukreja et al., 1990; Chopra et al., 1992). The FRs theory also explains, besides other multiple mechanisms, the initiation, progression, and aggravation of inflammation (Greenwald, 1991), as activation of phospholipase $A_2$ by inflammatory stimuli leads to release of inflammatory mediators and thereby reactive oxygen species. On the other hand, we previously reported the capability of CP to inhibit enzymatically generated LTs, from stimulated intact human neutrophils (Mansour and Agha, 1999). Therefore, the aforementioned properties of CP together with its immunosuppressive effect...
Effects of Captopril on the Inflammatory Mediators

Rats were randomly allocated into four groups of six to eight animals each. One group of rats was kept as normal, while the other three groups were subjected to inflammation. In the right hind paw, 0.4 mg of Freund’s complete adjuvant was sc injected. Nineteen days later, the back of each animal was shaved and 20 ml sterile air was sc injected under light ether anesthesia to form a dorsal air pouch. To the 4-day-old pouch, 0.6 mg of Freund’s complete adjuvant was inoculated (Tate et al., 1989). One group of animals subjected to inflammation was kept without treatment and served as control, while the remaining two groups were given CP. The drug was administered at daily doses of 1 and 10 mg/kg ip, starting from the day of adjuvant inoculation into the paw and up to the end of experimentation that lasted for 27 days. The dose of 100 mg/kg was not tested in MH, since the results between 10 and 100 mg/kg obtained in the FA model were not significantly different.

On the 28th day, blood samples were collected from all animal groups. Rats were then sacrificed and exudates of the granuloma pouches were withdrawn. The collected blood and exudate were used for the estimation of the chosen parameters (vide infra).

Determination of the inflammatory mediators and markers of oxidative stress. The blood samples from animals of both models were withdrawn from retroorbital veins. Each sample was divided into two portions. The first aliquot was placed in nonheparinized tube for serum separation and determination of LTB₄, IL-6 (Gauldie et al., 1990), PrSHs (Koster et al., 1986), and TBA-RS (calculated as malondialdehyde (MDA) and served as an indirect measure of lipid peroxide (LP)) (Uchiyama and Mihara, 1978). The second portion of blood was placed in a heparinized tube and used for estimation of erythrocytic SOD activity (Marklund and Marklund, 1974) and blood GSH (Beutler et al., 1996).

The collected exudates from the granuloma pouches of animals subjected to MH were centrifuged and their supernatants were used for estimation of LTB₄, IL-6, GSH (Ahmed et al., 1991), PrSHs, SOD, and TBA-RS. LTB₄ and IL-6 were determined by ELISA, using reagent kits of Neogen Corporation (Lexington, KY) and Genzyme Corporation (Cambridge, MA), respectively, while the remaining parameters were estimated spectrophotometrically.

Statistical analysis. Values are given as means ± SD. The level of statistical significance was taken at p < 0.05, using one-way ANOVA followed by Tukey and Kramer multiple comparisons test to judge the difference between various groups.

RESULTS

Effects of Captopril on Paw Edema Volume in Freund’s Arthritis

In control arthritic animals, the paw edema induced by Freund’s adjuvant inoculation was shown to be biphasic. An acute phase was evidenced on the 4th day postinoculation, followed by a delayed sustained chronic phase that reached a plateau starting from the 11th day and up to the 21st day (Fig. 1).

Long-term administration of CP at the selected doses, viz., 1, 10, and 100 mg/kg markedly inhibited the development of paw edema during the acute phase by 48, 60, and 83%, respectively, as compared to the control group. During the chronic phase of arthritis, CP produced a similar pattern of amelioration, as it suppressed the edema on the 21st day by 53, 60, and 82% at the aforementioned doses, respectively (Fig. 1).

Effects of Captopril on the Inflammatory Mediators

During the chronic phase of FA and MH, the serum level of LTB₄ has been increased by ca. 22–25%, as compared to
normal groups (Figs. 2A and 2B). Similarly, in both models of inflammation the serum IL-6 content has been elevated by about 24–25% (Figs. 3A and 3B).

The effect of CP on LTB₄ tended to be dose-related. At the low dose, 1 mg/kg, CP did not significantly affect LTB₄ in sera of both models (Figs. 2A and 2B), but it only reduced LTB₄ content of the exudate of MH by about 34% compared to control values (Fig. 2C). Treatment with CP at doses of 10 and/or 100 mg/kg resulted in normalization or even profound reduction in serum level of LTB₄ in FA (Fig. 2A) as well as in sera (Fig. 2B) and exudates (Fig. 2C) of animals subjected to MH.

Regarding its effect on IL-6, CP at the chosen doses significantly reduced the increased cytokine level in sera of both inflammatory models (Figs. 3A and 3B), reaching normal values. Likewise, in the exudates of MH, a remarkable decrease in IL-6 level has been demonstrated in CP-treated groups (Fig. 3C) compared to respective control values. No tendency of dose-related effect has been observed for CP on IL-6 levels.

**Effects of Captopril on the Markers of Oxidative Stress Induced by Inflammation**

A threefold increment in the content of TBA-RS in sera of Freund’s arthritic rats has been observed during the chronic phase of the disease compared to normal values (Fig. 4A). Similarly, in MH, a high TBA-RS content has been detected in
exudate reaching about 5.9 nmol MDA/ml (Fig. 4C), accompanied by a significant increase in its serum level (Fig. 4B). Long-term treatment with CP at the selected doses produced profound reduction in TBA-RS content in sera of FA (Fig. 4A). In the exudate of MH, CP at 1 and 10 mg/kg provoked pronounced inhibition of TBA-RS by ca. 64 and 60%, respectively, compared to the control group (Fig. 4C); but the drug did not significantly affect serum TBA-RS content in this model (Fig. 4B). It is noteworthy that some of the results in both models could vary because Freund’s adjuvant was administered as a single injection in case of FA, while it was administered twice in MH.

In the inflammatory exudates of animals subjected to MH, a detectable amount of SOD reaching 7 μg/ml has been found. The activity of the enzyme has been markedly impeded by long-term treatment with CP at doses of 1 and 10 mg/kg by 32 and 44%, respectively, compared to control values (Fig. 5C). However, in erythrocytes, its activity has not been significantly altered by induction of inflammation nor by treatment with CP (Figs. 5A and 5B).

The serum level of PrSHs has been markedly increased by 60% in FA (Fig. 6A) and by 43% in MH (Fig. 6B) compared to respective normal values. Treatment with CP produced more increase in serum thiols level in FA (Fig. 6A) but did not significantly alter its level in sera (Fig. 6B) or exudates (Fig. 6C) of animals subjected to MH.

Glutathione has been increased by about twofold in blood of animals subjected to either model compared to normal values (Figs. 7A and 7B). Administration of CP at the selected doses provoked reduction or even normalization of blood GSH level in FA (Fig. 7A). However, in blood (Fig. 7B) and exudates (Fig. 7C) of animals subjected to MH, the inhibitory effect of CP on GSH content did not reach significant levels.

**DISCUSSION**

Administration of CP at three dose levels elicited marked antiinflammatory effect in Freund’s adjuvant arthritic rats, as evidenced by profound suppression of paw edema development, during all phases of the disease.

Captopril is an ACE inhibitor and the beneficial effects of this class of drugs have been related to a decrease in angiotensin II (Breckenridge, 1988). However, ACE inhibitors possess several other modes of action, including inhibition of bradykinin (BK) (Breckenridge, 1988), as its enzymatic degradation can be brought by ACE (kininase II). BK is a mediator of early phases of acute inflammation, thus some investigators have examined the effect of CP on acute inflammation. They observed that CP at low doses (up to 5 mg/kg, single dose) potentiated inflammation, which is induced by stimuli that give
rise to BK (Decarie et al., 1996; Gaspar and Blazso, 1996), and they related this effect to ACE inhibitory action of CP (Gaspar and Blazso, 1996). However, at higher doses (up to 400 mg/kg, single dose), inhibition of inflammation was observed (Gaspar and Blazso, 1996) and was attributed to an effect of CP on microvasculature, as it enhanced capillary resistance, especially at high doses. Moreover, CP exerted significant protection against radiation injury in rat, as it reduced inflammation and fibrosis (Cohen et al., 1996). Thus, more attention has been paid to the effect of CP on chronic inflammation, where kinins do not play major role. CP succeeded in suppressing chronic polyarthritis of adjuvant arthritis in rat (Gaspar and Blazso, 1996), a model that resembles rheumatoid arthritis (RA) including immunologic reactivity and mediated by T cells (Crossley et al., 1989). Hence, it has been assumed that the immunosuppressive action of CP (Constantinescu et al., 1995; Gaspar and Blazso, 1996) and its protective effect on microvasculature (Gaspar and Blazso, 1996) might be partly responsible for reduction of chronic inflammation and, consequently, its antiarthritic action. Inhibition of ACE by CP could also be included, since ACE participates in T cell stimulation (Constantinescu et al., 1995).

In an attempt to further elaborate the mechanism of action of CP against chronic immunological inflammation, we studied its effect on certain inflammatory mediators in serum and/or inflammatory exudate of arthritic rats. During the chronic phase of FA and MH models, systemic levels of LTB₄ and IL-6 have been noticeably elevated in control groups, accompanied by high exudate content of both mediators. These effects are consistent with the role of LTB₄ and IL-6 in the pathology of arthritis. Among different LTs, LTB₄ is the strongest candidate of inflammation. Increased LTB₄ content has been demonstrated in inflammation, with marked relation to the severity of the disease (Crooks et al., 1996; Subbeyaz et al., 1996). It modulates immunologic and inflammatory responses, induces FRs generation, and enhances production of proinflammatory mediators, including cytokines (Ford-Hutchinson, 1990). Likewise, the serum level of IL-6 was markedly increased in adjuvant arthritis (Brauer et al., 1994) with a clear correlation with morphologic disease signs (Theisen-Popp et al., 1992) and kinetics of paw edema development (Leisten et al., 1990). IL-6 serum level was regarded as a useful parameter for monitoring the disease activity in arthritis (Leisten et al., 1990; Brauer et al., 1994), however, the synovial values reflect the disease activity more precisely (Brauer et al., 1994). Increment of cytokines was found in SF of different arthropathies, including RA (Dayer and Fenner, 1992; Bertazzolo et al., 1994); but the highest cytokine concentration found in SF of RA was that of IL-6, and its level was associated with the degree of synovitis (Bertazzolo et al., 1994). IL-6 is the most abundantly expressed cytokine in rheumatoid synovium, probably derived from type B synovial lining cells and fibroblasts, thereby it is released in response to insults that lead to joint damage (Madhok et al., 1993; Bertazzolo et al., 1994). A highly significant correlation between local IL-6 level and the severity of chronic arthritis in rat at days 14–28, but not in the early phase, has been shown (Brauer et al., 1994). Thus, IL-6 was demonstrated to be a good marker of arthritis, including FA (Leisten et al., 1990; Theisen-Popp et al., 1992) and antigen-induced arthritis (Brauer et al., 1994) in rat and RA (Bertazzolo et al., 1994).

The inhibitory effect of CP on the chronic phase of arthritis was accompanied by a marked reduction of the elevated local and systemic levels of LTB₄ and IL-6, with the effect being more pronounced on the local LTB₄ level. These effects would make an important contribution to the antiinflammatory activity of the drug. The alteration of LTB₄ profile is probably due to the inhibitory effect of CP on LTB₄ synthesis, while reduction of IL-6 could be regarded as a consequence of LTs inhibition and of the immunosuppressive action of CP.

Captopril inhibited LTB₄ synthesis, highlighted by its ability to inhibit LTA₄ hydrolase (Orning et al., 1990). In a previous study, we demonstrated that CP succeeded in reducing enzymatically generated LTs produced by stimulated human neutrophils, being more potent against LTB₄ (Mansour and Agha, 1999). It is noteworthy that the bifunctional catalytic traits of the zinc metalloenzyme LTA₄ hydrolase/aminopeptidase include generation of LTB₄ and a hyperalgesic substance as well as inactivation of analgesic opioid peptides (Griffin et al., 1992). Thus CP, as a LTA₄ hydrolase inhibitor, could reduce formation of both LTB₄ and an hyperalgesic substance as well as suppress hydrolysis of enkephalins (Griffin et al., 1992), which might add to the beneficial actions of CP against inflammation.

IL-6 is a multifunctional cytokine, and considered as an inflammation-promoting one (Philippe et al., 1997). It induces hypermetabolism and fever in rat, giving rise to the hypothesis that it is a circulating endogenous pyrogen (Rothwell et al., 1991). Thus, immunotherapy with anti-IL-6 has been proposed to be a promising approach to treatment of chronic inflammation (Brauer et al., 1994). CP significantly reduced IL-6 levels in serum and exudate of arthritic rats. This effect could be a consequence of its inhibitory effect on LTs, which are known to enhance cytokines release (Ford-Hutchinson, 1990), or attributed to its immunosuppressive property (Constantinescu et al., 1995). Indeed, certain immunosuppressive agents, such as cyclosporin A, succeeded in inhibiting chronic arthritis associated with normalization of IL-6 level (Leisten et al., 1990; Theisen-Popp et al., 1992; Brauer et al., 1994). Similar results have also been observed with some antirheumatics (Leisten et al., 1990; Theisen-Popp et al., 1992). However, few reports proposed a striking contradictory feature for IL-6: that it possesses some antiinflammatory activity (Gonzalez et al., 1994).

In control arthritic animals, a significant amount of SOD has been detected in the inflammatory exudate; meanwhile, systemic levels of GSH, PrSHs, as well as LP have been increased as compared to normal groups.

Systemic LP, measured as TBA-RS, rose during adjuvant
arthritis (Agha and Gad, 1995; Agha et al., 1999). LP produces lysosomal destruction leading to tissue damage, hence its close association with aggravation of arthritis (Halliwell, 1994). It could, therefore, account for or be a reflection of the severity of the biochemical aspects of arthritis. The observed increment of systemic PrSHs and GSH stems most probably as a consequent defensive mechanism against the arthritic insult, to down-regulate the inflammatory response by inhibiting peroxides overshooting, since thiols are involved in destruction of peroxides formed during phagocytosis by granulocytes (Hall et al., 1984). Enhancement of the GSH systemic level could also be a result of NADPH overproduction by the HMP shunt, thereby glutathione reductase uses NADPH to reduce glutathione disulphide to GSH (Schaufstatter et al., 1985). Increased mobilization of endogenously synthesized GSH from liver to other sites, such as erythrocytes or inflammatory sites, to satisfy their GSH requirement during inflammation could be another explanation. It merits mention that, during inflammatory disorders, both enhancement (Igari et al., 1982) as well as exhaustion (Imadaya et al., 1988) of endogenous FR scavengers have been reported. However, in either case, effective anti-inflammatory drugs produce their protective effects by normalization of the altered profile of FRs, their products, and consequently their scavengers.

Captopril ameliorated the aggravated oxidative stress induced by arthritic insult. It lowered LP, enhanced systemic PrSHs, and normalized the levels of endogenous FR scavengers that have been elevated, as a defensive compensatory mechanism, during arthritis.

Captopril was shown to exert a protective effect against FR-generating models, such as I/R injury (Birincioglu et al., 1997), and diquat-induced oxidative damage (Bhuyan et al., 1992). It reduced tissue MDA or TBA-RS formation both in vitro and in vivo (Chopra et al., 1992; Buyukgebiz et al., 1994; de Cavanagh et al., 1997) and impeded elevated breath pente, which is a product of lipid peroxidation, in chronic heart failure patients (Sobotka et al., 1993). Generally, the level of LP can be reduced by amelioration of the pathological disorder or by use of FR scavengers.

The observed increase in the systemic PrSHs in CP treated groups could be attributed to the protective effect of CP on microvasculature (Gaspar and Blazso, 1996). By increasing capillary resistance, CP produces reduction in plasma protein extravasation, leading to increase in systemic albumin, which constitutes about 90% of PrSHs (Thomas and Evans, 1975). The -SH moiety of CP might also share part in this effect, as it could be estimated in serum as part of the determined thiols.

Captopril inhibited the activity of SOD in inflammatory exudate but not in erythrocytes of arthritic animals subjected to MH. The effect was diverted to the inflammatory site and could be a consequence to less availability of the superoxide anion, as result of the antiarthritic effect of the drug; but we could not certify a direct effect to CP on this radical. PMN leukocytes present in SF produce the superoxide radical, possessing an apparent salutary function of killing the engulfed bacteria (Greenwald, 1991; Winrow et al., 1993). However, as an untoward side effect, it produces degradation of polymers, as polysaccharides, so the reaction is viewed as an in vivo mechanism of SF destruction in inflamed joints. This radical is eliminated by SOD (Greenwald, 1991; Winrow et al., 1993), thus its reduced generation results in reduction of SOD, which could give a reasonable explanation of the observed effect of CP on SOD in the inflammatory exudate. Indeed, in vitro, CP reduced the amount of superoxide anion detected after activation of neutrophils with zymosan (Chopra et al., 1992).

Captopril normalized the systemic level of GSH in FA, which could be a consequence of its antiarthritic action, leading to modulation of oxidant status. GSH, in addition to its powerful scavenging property, reduces disulphides, thus GSH might be consumed by CP, because in the body much of CP is carried as disulphides with an endogenous -SH group such as GSH (Heel et al., 1980). On the other hand, these disulphides serve as depot form of CP and function as a recyclable anti-oxidant agent (Chopra et al., 1989), an effect that might be relevant in its therapeutic usefulness.

Captopril can actually protect animals against damage induced by arthritic insults. This action could probably be mediated via inhibition of local and systemic LTβ, thereby leading to reduction of IL-6, as LTβs enhance cytokines release. A FR scavenging property could not be excluded, as enhancement of systemic PrSHs with normalization of the altered profile of FR products and scavengers have been observed in CP-treated arthritic groups. However, the effect of CP on the oxidative stress could be a consequence of its antiarthritic effect. Thus, the data strengthen the argument that CP can be used in therapy of arthritic diseases, and if confirmed by further toxicological and clinical studies, the present study could afford a basis for long-term use of CP in patients suffering from inflammatory conditions.

REFERENCES


