Verapamil Inhibits Elastase Release and Superoxide Anion Production in Human Neutrophils

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Received May 16, 1997; accepted September 29, 1997

In response to activation of phagocytic cells and during inflammatory disorders, some proteases and very reactive oxygen species are produced. These proteases and oxidants are involved in many diseases like tissue injury or atherosclerosis. We have shown *in vitro* that verapamil, a calcium channel blocker, had antielastase and antioxidant properties. This drug inhibited the release of elastase by neutrophils in a dose-dependent manner when these cells were stimulated by phorbol-myristate-acetate (PMA), by *N*-formyl-methionyl-leucylphenylalanine (fMLP) and by the calcium ionophore A23187 (Ca.I). In addition, verapamil inhibited superoxide anion when human neutrophils were stimulated by PMA, fMLP, dioctanoylglycerol (DiC8), Ca.I or by opsonised zymosan (OZ). Verapamil did not act by scavenging elastase or oxidants as demonstrated in cell-free models which showed no direct antielastase or antioxidant effect involved by verapamil. Superoxide anion and elastase inhibition by verapamil has been considered to the mobilization of cytosolic calcium and inhibition of protein kinase C. The results suggest that verapamil might contribute help the development and progression of atheroma where oxidants and elastase are involved.

Key words verapamil; PMN; superoxide anion; elastase; cytosolic calcium

Neutrophils play a central role both in host defense against invading microorganisms and in induction and regulation of the inflammatory process. The cells have been shown to liberate potent proteinases (elastase, collagenase) and to generate some oxygen-derived species such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). Then, the hydroxyl radical ($OH^\cdot$) is formed by the Haber–Weiss reaction. Activation of neutrophils also induced release of the azurophil granule protein, myeloperoxidase, which reacts with H_2O_2 and produces hypochlorous acid.¹⁾

Proteinases liberated by polymorphonuclear leukocytes (PMNs) are stored within granules and neutrophil elastase has been shown to degrade all components of the extracellular matrix with production of elastin-derived peptides but also to cleave a variety of key proteins and even to attack intact cells.^{2,3)} This enzyme is thought to play a role in the pathogenesis of many diseases such as tissue injury or atherosclerosis.^{4,5)}

It has been shown that oxidants are involved in the generation of tissue injury in which $OH^\cdot$ is particularly dangerous and HOCl is the most powerful neutrophil oxidant known, cytotoxic and can destroy structural proteins.⁶⁾ Oxygen species have also been implicated in the pathogenesis of atherosclerosis.⁷⁾ Several investigators,⁸⁾ have shown that superoxide anion producing to the biological modification of low density lipoprotein (LDL) can contribute to the progression of atheroma *via* oxidized LDL.

After exposure to certain stimuli such as phorbol-myristate-acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), opsonized zymosan (OZ), dioctanoylglycerol (DiC8) and calcium ionophore A23187 (Ca.I), the respiratory burst in neutrophils is activated. PMA, a phorbol ester, directly activates protein kinase C (PKC).⁹⁾ fMLP activates phospholipase D (PLD) and phospholipase C (PLC) with the production of inositol-tri-phosphate (IP_3) and diacylglycerol (DAG).^{10,11)} DAG and IP_3 are known to activate PKC and to

mobilize intracellular calcium stores, respectively. OZ activates phospholipase A2 (PLA2),¹²⁾ which is a Ca^{2+} dependent enzyme. DiC8, a synthetic diacylglycerol, activates PKC and does not induce a change in $[Ca^{2+}]$.¹³⁾ Ca.I increases cytosolic calcium.¹⁴⁾

The production of elastase is also induced by some stimuli such as PMA, fMLP and Ca.I,¹⁵⁾ but not by OZ.¹⁶⁾ Our results confirm that OZ is a relatively poor stimulator of granulocyte degranulation.

Several studies have reported that calcium channel blockers such as nifedipine and nisoldipine, which are used in hypertension or angina, have antioxidant properties,⁸⁾ *via* an unknown antioxidant mechanism.^{14,17,18)}

The aim of this study was to evaluate the action of verapamil, a calcium channel blocker, on the production of superoxide anion from neutrophils subjected to different stimuli in order to clarify its antioxidant mechanism. Since leukocyte elastase activity is involved in many processes and the explanations of this activity suggested by some authors^{15,19)} differ, we also studied the action of verapamil on elastase release.

MATERIALS AND METHODS

Reagents Verapamil, specific substrate methoxy-Suc-(Ala)₂-Pro-Val-pNA, phenylmethylsulfonyl fluoride, ferricytochrome C, PMA, fMLP, OZ, DiC8, Ca.I and superoxide dismutase were obtained from Sigma and elastase from Biosys.

Isolation of Human Polymorphonuclear Neutrophils (PMNs) PMNs were purified from fresh heparinized venous blood of healthy human subjects. Briefly, PMNs were isolated by a gradient density technique followed by isotonic ammonium chloride haemolysis.²⁰⁾ Cells isolated by this technique were viable as shown by trypan blue exclusion and cells were suspended in Hank's Hepes (HH) pH 7.4 buffer.

Cellular Viability after Verapamil Reaction In order

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to control the cellular viability after verapamil, application of a viability test by trypan blue exclusion was performed at the end of every cellular experiment. Trypan blue is able to enter into dead cells and the percentage of dead PMNs is counted in a Thomas chamber.

Human Neutrophil Elastase Release Neutrophils were incubated for 15 min at a concentration of $2 \times 10^6/\text{ml}$ in HH buffer pH 7.4 in the presence of increasing concentrations of verapamil at 37°C .²¹⁾ Then, PMA (160 nmol/l), fMLP (1 $\mu\text{mol/l}$) or Ca.I (4 $\mu\text{mol/l}$) was added and the reaction mixtures incubated for 30 min at 37°C . After incubation, the tubes were centrifuged for 5 min at 4°C . Cell-free supernatants were assayed for elastase activity. Briefly, 400 μl supernatant was added to 400 μl specific substrate methoxy-Suc-(Ala)₂-Pro-Val-pNA (0.5 mmol/l in 0.1 mol/l HEPES-0.5 mol/l NaCl pH 7.5 buffer) in a final volume of 1 ml. After a 5 min incubation at 37°C , the reaction was stopped with phenylmethylsulfonyl fluoride (PMSF) and absorbance was measured at 405 nm in a spectrophotometer (Kontron Uvikon 860). Elastase concentration was obtained from a standard curve using various concentrations of reagent grade elastase. Results were expressed as the percentage of elastase activity inhibited by verapamil.

Direct Inhibition of Human Leukocyte Elastase (HLE) Neutrophils were incubated for 30 min at a concentration of $2 \times 10^6/\text{ml}$ in HH buffer in the presence of fMLP (1 $\mu\text{mol/l}$). Then, the tubes were centrifuged at 4°C for 5 min in order to collect elastase from the supernatant and 400 μl supernatant was added to 100 μl of increasing concentrations of verapamil at 37°C for 15 min. After incubation, 400 μl specific substrate (methoxy-Suc-(Ala)₂-Pro-Val-pNA) was added to reaction mixtures in a final volume of 1 ml and allowed to stand for 5 min. The reaction was stopped with PMSF (0.1 mol/l) and absorbance was measured at 405 nm in a spectrophotometer.

Superoxide Anion (O_2^-) Released by Human Neutrophils Superoxide anion (O_2^-) production by human PMNs stimulated by PMA (160 nmol/l) or fMLP (1 $\mu\text{mol/l}$) or OZ (0.3 mg/ml) or DiC8 (20 $\mu\text{mol/l}$) or Ca.I (4 $\mu\text{mol/l}$) was measured by reduction of ferricytochrome C.²²⁾ 1.5×10^6 PMNs, stimulated by one of these five agents, were incubated for 5 min at 37°C in the presence of increasing concentrations of verapamil and 0.3 mg ferricytochrome C in HH buffer pH 7.4. After centrifugation at 4°C , the absorbance of the supernatants was measured with a Kontron Uvikon 860 spectrophotometer against a reference cuvette containing 310 U superoxide dismutase. The difference spectrum was monitored at 550 nm and the amount of O_2^- was calculated using an extinction coefficient $E_{550} = 21.1 \text{ mmol/l} \times \text{cm}^{-1}$. The results are expressed in nmol $\text{O}_2^-/10^6$ PMNs per 5 min.

Scavenging of Superoxide Anion (O_2^-) Generation of superoxide anion by the hypoxanthine-xanthine oxidase system was carried out essentially as described in elsewhere.²³⁻²⁴⁾

Statistical Analysis Results are expressed as means $\pm$ standard error of the means (S.E.M.). The significance of any differences was determined using Wilcoxon's test and p values below 0.05 were considered statistically significant.

RESULTS

Cellular Viability after Verapamil Reaction Verapamil used in the range of concentrations from 10 to 500 μM had no effect on cellular viability determined by trypan blue exclusion.

Effect of Verapamil on Neutrophil Elastase Release The amount of elastase release by stimulated PMNs was 20 nmol/l, 13.25 nmol/l and 8.45 nmol/l in PMA, fMLP and Ca.I system respectively.

When verapamil was incubated with human neutrophils, the release of elastase was inhibited by this drug in a concentration-dependent manner, as shown in Fig. 1. The concentration of this drug giving 50% control activity (IC_{50}), was 75 $\mu\text{mol/l}$, 60 $\mu\text{mol/l}$ and 31 $\mu\text{mol/l}$ in PMA, fMLP and Ca.I stimulated cells respectively. In the cell-free system, verapamil did not directly inhibit HLE.

Effect of Verapamil on Superoxide Anion (O_2^-) Vera-

Fig. 1. Effect of Verapamil on Release of Elastase by PMA-, fMLP- and Ca.I-Activated Neutrophils

Results are expressed as the percentage of elastase release inhibited by verapamil. Values are the means $\pm$ S.E.M. of 5 separate experiments. * Significantly different from verapamil-free control ($p < 0.05$).

Fig. 2. Inhibition by Verapamil of O_2^- Production by OZ- or DiC8- Stimulated Human Neutrophils

Stimulated PMNs were incubated with increasing concentrations of verapamil at 37°C for 5 min. The production of O_2^- was measured as described in Methods. The results are expressed as % of O_2^- production inhibited by verapamil. Values are the means $\pm$ S.E.M. of 4 separate experiments. * Significantly different from verapamil-free control ($p < 0.05$).

Fig. 3. Inhibition by Verapamil of O_2^- Production by fMLP-, PMA- or Ca.I- Stimulated Human Neutrophils

Stimulated PMNs were incubated with increasing concentrations of verapamil at 37 °C for 5 min. The production of O_2^- was measured as described in Methods. The results are expressed as % of O_2^- production inhibited by verapamil. Values are the means $\pm$ S.E.M. of 4 separate experiments. * Significantly different from verapamil-free control ($p < 0.05$).

pamil was able to inhibit O_2^- production by PMA-, fMLP-, OZ-, DiC8- and Ca.I- stimulated human neutrophils in a concentration-dependent manner as shown in Figs. 2 and 3 respectively, with an IC_{50} of 200 μ mol/l, 50 μ mol/l, 280 μ mol/l, 100 μ mol/l and 75 μ mol/l in the case of PMA, fMLP, OZ, DiC8 and Ca.I respectively. In the cell-free system, verapamil did not scavenge superoxide anions directly.

DISCUSSION

Stimulation of neutrophils results in an oxidative burst and release of proteases. In this process, reactive oxygen species and elastase are liberated. Excess oxidants and elastase can be highly toxic and there are many steps in the pathogenesis of a number of diseases such as myocardial injury,²⁵ emphysema,²⁶ or atherosclerosis,⁴ that calcium antagonists may be expected to affect. In contrast to Werner *et al.*,¹⁵ who have shown that verapamil was ineffective in suppressing elastase release at concentrations up to 100 mmol/l, our studies have demonstrated that verapamil caused a concentration-dependent inhibition of elastase release from human neutrophils *in vitro*. These results are in keeping with the findings of Kokot *et al.*¹⁹ However in our PMA system, lower concentrations of verapamil inhibited release of elastase whereas Kokot *et al.* used higher concentrations to inhibit these responses. The difference may be due to the fact that Kokot *et al.* used a different method.

In this study, the IC_{50} obtained in PMA, fMLP and Ca.I systems was 75 μ mol/l, 60 μ mol/l and 21 μ mol/l, respectively. Verapamil did not act by inhibiting elastase because, in a cell-free model, elastase is not directly inhibited by this drug. Moreover, verapamil effects are not due to cytotoxicity as demonstrated by the trypan blue exclusion test. The mechanism of this antielastase activity might be explained by inhibition of calcium mobilization because elastase production was more important in fMLP and Ca.I systems. On the other hand, in view of the elastase control values, fMLP and Ca.I both produce less powerful stimuli than PMA. However, inhi-

bition of PKC cannot be excluded because in the PMA system, elastase generation was decreased.

Also in this paper, verapamil has been shown to have antioxidant properties towards O_2^- . Verapamil was clearly shown to inhibit PMA-, fMLP-, OZ-, DiC8- and Ca.I- stimulated oxygen radical production from neutrophils, as reported by several investigators for some calcium channel blockers.^{8,27} In the cell-free model, we have shown that this drug did not act by scavenging superoxide anion. The IC_{50} of 200 μ mol/l observed for verapamil inhibition of O_2^- generation by PMA was lower than the IC_{50} of 300 μ mol/l reported by Irita *et al.*,¹⁸ but not lower than the IC_{50} of 88 μ mol/l reported by Weisdorf *et al.*¹⁴ The mechanism of this antioxidant activity in neutrophils can be explained by several hypothesis. On the one hand, an effect on the mobilization of cytosolic calcium might be considered because verapamil inhibited oxygen radical production by fMLP stimulated neutrophils with an IC_{50} lower than in PMA-, DiC8- and OZ-stimulated neutrophil systems. This effect might be explained by the pathway of fMLP. This stimulus is known to activate PLC which produces two species: DAG and IP_3 . The former activates PKC and the latter mobilizes intracellular calcium stores. This cellular antioxidant mechanism of verapamil is in agreement with the results of a few authors like Rosales *et al.*,²⁸ who found that several calcium channel blockers inhibited the movement of Ca^{2+} from cytosol to intracellular stores. In addition, Weisdorf *et al.*¹⁴ suggested that the inhibitory effect of verapamil on O_2^- generation from stimulated PMNs involved inhibition of calcium mobilization. Moreover, Ca.I is known to increase cytosolic calcium. These data could explain the inhibition of verapamil in these models. On the other hand, an inhibition of PKC cannot be excluded in keeping with the results of Irita *et al.*¹⁸ because verapamil inhibited oxidants in PMA- and DiC8-stimulated neutrophils. PMA is known to activate PKC directly and to enhance the calcium affinity of the kinase. Moreover, DiC8 resulted in activation of PKC. So, in this study, the results suggested that verapamil affected superoxide anion production by inhibition of calcium mobilization and/or an inhibition of PKC.

With these antioxidant properties, verapamil might reduce oxidized low density lipoprotein (LDL) production by inhibition of oxidant generation from neutrophils. Indeed, oxygen species may contribute to biological modification of LDL, resulting in an enhanced uptake of modified LDL by macrophages, foam cell formation and atherogenesis.⁴

Also, inhibition of elastase by verapamil may be physiologically important in preventing excess tissue damage and in reducing elastin-derived peptide production. These peptides have been shown to contribute to some adverse biological activities such as proliferation of smooth muscle cells which plays a key role in atherosclerosis.²⁹

With these antielastase and antioxidant properties, verapamil might prevent tissue damage during inflammatory diseases or attenuate the development and progression of atheroma by inhibition of elastin-derived peptide production and inhibition of oxygen species.

Acknowledgement. We thank S. Lebegue-Battez for her technical assistance.

REFERENCES

- 1) Halliwell B., *Amer. J. Med.*, **91**(3C), 14S—22S (1991).
- 2) Weiss S. J., *N. Engl. J. Med.*, **320**, 365—376 (1989).
- 3) Iwamura H., Moore A. R., Willoughby D. A., *Biochim. Biophys. Acta.*, **1156**, 295—301 (1993).
- 4) Mitchinson M. J., Ball R. Y., *Lancet.*, **18**, 146—149 (1987).
- 5) Fulop T., Jr., Jacob M. P., Varga Z. S., Foris G., Leovey A., and Robert L., *Biol. Biophys. Res. Comm.*, **141**, 92—98 (1986).
- 6) Wallaert B., Gressier B., Aerts C., Mizon C., Voisin C. and Mizon J., *Am. J. Respir. Cell. Mol. Biol.*, **5**, 437—444 (1991).
- 7) Lehr H. A., Seemuller J., Hubner C., Menger M.G., and Messmer K., *Arterioscler. Thromb.*, **13**, 1013—1018 (1993).
- 8) Sakamoto S., Fujie K., Handa H., *Arzneim. Forsch. Drug. Res.*, **41**, 781—785 (1991).
- 9) Nishizuka Y., *Science*, **233**, 305—315 (1986).
- 10) von Tscharnier V., Prod'homme B., Baggiolini M., and Reuter H., *Nature* (London), **324**, 369—372 (1986).
- 11) Kessels R. G. C., Ross D., Verhoeven A. J., *J. Biol. Chem.*, **266**, 23152—23156 (1991).
- 12) Maridonneau-Parini I., Tringale M., Tauber A. I., *J. Immunol.*, **137**, 2925—2929 (1986).
- 13) Kawada M., Edashige K., Watanabe Y., Yasuda T., and Utsumi K., *Physiol. Chem. Phys.*, **25**, 237—252 (1993).
- 14) Weisdorf D. J., Thayer M. S., *J. Lab. Clin. Med.*, **114**, 260—265 (1989).
- 15) Werner U., Seitz O., Szelenyi I., *Agent Action*, **38**, C112—C114 (1993).
- 16) Turner N. C., Wood L. J., Szelenyi I., *Eur. Respir. J.*, **7**, 934—940 (1994).
- 17) Karnad A. B., Hartshorn K. L., Tauber A. I., *Agent Action*, **31**, 79—85 (1990).
- 18) Irita K., Fujita I., Takeshige K., Minakami S., and Yoshitake J., *Biochem. Pharmacol.*, **35**, 3455—3471 (1986).
- 19) Kokot K., Teschner M., Schaefer R. M., and Heidland A., *Mineral Electrolyte Metab.*, **13**, 133—140 (1987).
- 20) Cabanis A., Gressier B., Lebegue S., Brunet C., Dine T., Luyckx M., Cazin M. and Cazin J. C., *APMIS*, **102**, 119—121 (1994).
- 21) Gressier B., Lebegue S., Brunet C., Dine T., Luyckx M., Cazin M. and Cazin J. C., *Int. J. Pharm.*, **104**, 151—156 (1994).
- 22) Cohen H. J., Chovanec R. E., *J. Clin. Invest.*, **61**, 1081—1087 (1978).
- 23) Aruoma O. I., Halliwell B., Hoey B. M., and Butler J., *Free Radical Biol. Med.*, **6**, 593—597 (1989).
- 24) Gressier B., Cabanis A., Lebegue S., Brunet C., Dine T., Luyckx M., Cazin M. and Cazin J. C., *Pharm. World Sci.*, **17**, 76—80 (1995).
- 25) Flaherty J. T., *Amer. J. Med.*, **91**(3C), 79S—85S (1991).
- 26) Wallaert B., Gressier B., Marquette C. H., Gosset P., Remy-Jardin M., Mizon J. and Tonnel AB., *Am. Rev. Respir. Dis.*, **147**, 1537—1543 (1993).
- 27) Wong K., Kwan-Yeung L., *Toxicol. Appl. Pharmacol.*, **107**, 514—525 (1991).
- 28) Rosales C., Brown E. J., *J. Biol. Chem.*, **267**, 1443—1448 (1992).
- 29) Nilsson J., Sjolund M., Palmberg L., Von Euler A. M., Jonzon B., and Thyberg J., *Atherosclerosis*, **58**, 109—122 (1985).