POLYMPHONUCLEAR LEUKOCYTE MEDIATED OXIDATIVE INACTIVATION OF ALPHA-1-PROTEINASE INHIBITOR: MODULATION BY NITRIC OXIDE

Mohammad Muzaffar Mir*, Abdul Rashid Khan*, Nazir Ahmad Dar* and Mohammad Salahuddin **

Departments of Clinical Biochemistry* & Immunology**, Sher-I- Kashmir Institute Medical Sciences, Soura, 190 011, Srinagar (J&K) India.

ABSTRACT

Alpha-1-proteinase inhibitor activity was studied in presence of resting and activated polymorphonuclear leucocytes. Four different agonists; phorbol myristic acetate, N-formyl-methionyl-leucyl-phenylalanine, opsonised zymosan and arachidonic acid decreased the inhibitor activity by 23.3%, 20%, 12% and 16.6% respectively. The inhibitor activity was protected by using various free radical scavengers. Catalase and superoxide dismutase both restored activity by about 18 %, mannitol by 13% and sodium azide by 17.3%. The inhibitor activity was also protected significantly by pretreatment of polymorphs with L-Arg, a precursor of nitric oxide, before activation. L-Arg was also observed to suppress the generation of superoxide and hydroxyl radical appreciably. The nitric oxide synthase inhibitor, aminoguanidine drastically inhibited the nitrite release and reversed the protection offered by L-Arg to the inhibitor activity. Our results indicate a multifactorial nature of the inactivation process, the culprit species being superoxide, hydrogen peroxide, hydroxyl radical and hypohalides. Nitric oxide seems to scavenge the superoxide radical directly after its formation rather than inhibiting its generation by NADPH oxidase as was believed earlier.

KEY WORDS

Alpha-1-proteinase inhibitor, oxidative inactivation, polymorphonuclear leucocytes, nitric oxide, reactive oxygen species, reactive nitrogen metabolites.

INTRODUCTION

Polymorphonuclear leucocytes (PMNLs), as a part of their cytotoxic activity, release reactive oxygen species (ROS) in their surrounding medium (1,2,3,4). The ROS include superoxide (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and hypohalides. Nitric oxide and superoxide combine to form peroxynitrite, a relatively long lasting oxidant and hydroxyl radical, a much lethal species (5,6,7). ROS modulate Ca^{2+} signaling and protein phosphorylation events and thereby function as regulators of various biological processes including gene expression, cell growth, differentiation, chemotaxis, apoptosis and defense mechanisms (8,9,10).

The role of NO is diverse varying from an endogenous modulator of airway function to a pro-inflammatory and immunomodulatory mediator in patho-physiological conditions (5,11,12). Clancy *et al.* (13) reported the

inhibition of O_2^- production by NO whereas Beckman and Crow (14) showed the formation of more lethal and long lasting species like peroxynitrite by the interaction of NO with O^- . A variety of cell types including PMNL, which simultaneously generate nitric oxide and superoxide (15), in addition might produce hydroxyl radicals from the decomposition of peroxynitrite (16). NO has been reported to enhance free radical generation at lower concentrations while as at higher concentrations it inhibits the same (17,18,19). ROS damage and inactivate many biomolecules including alpha-1-proteinase inhibitor (16,20). Alpha-1-proteinase inhibitor (API) also called as alpha-1-antitrypsin, inhibits a diverse range of serine proteinases including pancreatic and neutrophil elastase (21,22). However it is most specific for neutrophil elastase with an association constant of $6.5 \times 10^7 M^{-1}$ (23). An imbalance in the elastase/API ratio in the lower respiratory tract is the most widely accepted hypothesis for alveolar destruction, loss of lung elasticity and emphysema (24). Previously we reported increased superoxide generating capacity of PMNL from human smokers (25) and we also observed unaltered total API activity in apparently healthy smokers (unpublished results).

In this study we made an attempt to study the

Author for correspondence :

Dr M Muzaffar Mir

Department of Clinical Biochemistry
Sher-I-Kashmir Institute of Medical Sciences
Soura, 190 011, Srinagar (J&K), India.
E. mail : mirmuzaffar@sify.com

inactivation of API by the activated PMNLs using four agonists, phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (FMLP), opsonized zymosan (OZ) and arachidonic acid (AA). The role of NO in the inactivation process was also studied by including the L-Arg (a precursor of NO) in the activated PMNL suspensions.

MATERIALS AND METHODS

Human serum albumin, cytochrome-C (type III), porcine pancreatic elastase (EC 3.4.21.36), horseradish peroxidase (EC 3.4.21.4), superoxide dismutase (EC 1.15.1.1), fumed silica, dithiothreitol (DTT), dimethyl sulphoxide, triton-X-100, N-succinyl-L-alanyl-L-alanyl-L-para-nitroanilide (SLAPN), dextran-500, L-Arg, trypan blue, aminoguanidine, 4-aminophenazone, phorbol-12-myristate, N-formyl-methionyl-leucyl-phenylalanine, arachidonic acid, zymosan, DEAE-cellulose, naphthyl ethylene diamine dihydrochloride (NEDD), acrylamide, N,N'-methylene bisacrylamide, ammonium persulphate, sodium dodecyl sulphate, N,N,N',N'-tetramethyl ethylenediamine, Krebs ringer phosphate buffer (KRPB), Hanks balanced salt solution (HBSS) and brilliant blue R were purchased from Sigma (St. Louis, MO, USA). The other chemicals of analytical grade were obtained from standard Indian sources.

Isolation and purification of API

API was purified from human plasma according to the procedure recommended by Glaser *et al.* (26) with some modifications. 200 ml of freshly separated human plasma was obtained from blood bank of Sher-I-Kashmir Institute of Medical Sciences (SKIMS) and was diluted with equal volumes of 0.1M tris buffer pH 8.5. DTT and fumed silica were added to final concentration of 25mM and 2.5% respectively and the contents were stirred at 25°C for 24 hours on a magnetic stirrer. The DTT treated plasma was salted out in 50% ammonium sulphate at 4°C and was equilibrated overnight. The precipitated plasma proteins were centrifuged at 500g for one hour in a refrigerated centrifuge. The supernatant was removed and dialyzed against 10 mM sodium phosphate buffer pH 7.6. The dialysate was loaded on a DEAE-cellulose column (15x2.5 cm) equilibrated with 10 mM sodium phosphate buffer pH 7.6 to a concentration of 40 mg protein / ml resin. The column was washed with equilibrating buffer until the O.D. of the eluted buffer at 280 nm approached zero. API was eluted with a gradient of 0.01M sodium phosphate and 0.2M NaCl. 3.0 ml fractions were collected and fractions having a specific activity greater than 0.5 were pooled. The pooled fractions were concentrated and subjected to gel chromatography on Sephadex G-25. The fractions containing API were again pooled and lyophilized. The specific extinction co-efficient of 0.5 (at 280 nm) was used to determine protein concentration of purified

product. The purity of isolated proteins was judged by SDS-PAGE.

Elastase inhibitory activity (EIA) of API

EIA was determined according to the method of Beatty *et al.* (27) using the synthetic substrate, SLAPN. The API inhibits the hydrolysis of SLAPN by elastase and the degree of this inhibition by API gives elastase inhibitory activity. 5.0 ml of stock elastase solution (5 mg/ml in 0.05M tris -NaCl, pH 8.0) was mixed with 100 µl of 0.2 M tris-HCl, pH 8.0 in a 1.0 ml cuvette. The volume was adjusted to 1.0 ml by addition of 0.05 M tris-HCl pH 8.0 and 20 ml of stock SLAPN (30 mg/ml in DMSO) was added. Elastase activity was measured as the change in the absorbance per minute at 405 nm.

$$\text{Elastase activity } (\mu\text{mol/min/ml}) = \frac{\text{Assay volume (ml)} \times \Delta A_{405}}{10.5 \times \text{sample volume (ml)} \times \text{Time (min)}}$$

Where 10.5 is the molar extinction coefficient of para-nitroanilide at 405nm liberated on hydrolysis of substrate. For EIA, 20 µl of sample (API) was mixed with 100 µl of 0.2 M tris HCL buffer pH 8.0 in 1 ml cuvette and was treated exactly as described above for the elastase activity. The residual elastase activity was determined and was used to calculate EIA as under:

$$\text{EIA} = \text{Elastase activity} - \text{residual elastase activity.}$$

Isolation of PMNLs

The PMNLs were isolated according to the method described earlier (25). Ten ml random blood sample was collected in a heparinized syringe (from apparently healthy and nonsmoker volunteers, mostly SKIMS employees) and mixed with 6% (w/v) Dextran-500 in normal saline in ratio of 4:1 and allowed to stand for 60 min. at room temperature. The supernatant was aspirated into a plastic tube and centrifuged at 275g for 10 min. The resulting pellet was resuspended in 8.0 ml of KRPB and layered over 3.0 ml cold histopaque-1077 and centrifuged for 20 min at 100 g. The supernatant was aspirated up to approximately 0.5 cm above the PMNL layer and the sides of the tube were cleaned with a sterile and clean cotton swab to remove any contaminating adherent cells. The RBCs present in the cell pellet were lysed by adding 9 volumes of distilled water to this, followed by vigorous shaking for 30 sec. using a cyclomixer. Osmolarity was restored by adding 1.0 ml 9% NaCl. The cell suspension was again centrifuged at 275 g for 5min and the resultant pellet was washed three times with KRPB and finally suspended in 1-2 ml of KRPB. Cells were counted and viability was established by trypan blue test. The experiments involving PMNL were organized in such a way that the whole exercise was completed in one working day without the overnight disruption.

PMNL activating agents

Four different agonists were used for the activation of PMNL. Stock solutions were prepared and divided in small aliquots and stored at requisite temperatures as described below.

1. PMA: Stock solutions (1 mg/ml) was made in DMSO and stored at -20°C .
2. AA: Stock solution (2.6 mM) was made in DMSO and stored at -20°C .
3. FMLP: Stock solution of FMLP (10 mM) in DMSO was prepared and stored at -20°C .
4. Oponised zymosan (OZ): 15 mg of zymosan was boiled in 1.0 ml of distilled water till a paste was formed. After cooling 1.0 ml human serum was added and the contents were incubated at 37°C for 30 min. Zymosan was centrifuged at 300g for 10 minutes at room temperature and resuspended in phosphate buffer saline for a final concentration of 15 mg/ml. The suspension was divided into 0.2 ml aliquots and stored at -70°C .

Estimation of superoxide

The amount of superoxide generated was estimated by measuring the SOD-inhibitable reduction of cytochrome C as described earlier (25). 2×10^6 cells/ml suspended in KRPB were pipetted into three clean plastic tubes each containing cytochrome C solution (100 μM in KRPB). SOD was added to one of the tubes to a final concentration of 100 $\mu\text{g}/\text{ml}$. After incubation for 5 min at 37°C , the stimulants at appropriate concentrations were added to the other tube. All the tubes were shaken and incubated at 37°C for 10 min in water bath. After incubations, the tubes were immediately transferred to a beaker containing melting ice and were centrifuged to remove the cell debris at 400g for 5 min in a refrigerated centrifuge. Cytochrome C reduction was measured by recording the absorbance at 550 nm against a reference solution containing SOD. The absorbance was multiplied by a factor of 47.7 to give superoxide concentration in n moles/ 2×10^6 cells/ml/10min.

Determination of hydroxyl radical

The hydroxyl radical generation was measured according to the method described by Greenwald *et al.* (28). The method is based on the degradation of deoxyribose by the hydroxyl radical to a chromogen which is read at 532 nm. To freshly isolated PMNL (2×10^6 cells) suspended in 0.2 ml HBSS buffer was added 0.5 ml of deoxyribose solution (20 mM) and 0.1 ml ferric-EDTA reagent. The reaction was started by adding optimized concentration of activator. The volume was made to 1.0 ml by HBSS. A blank, in which activator was omitted was also run. The reaction mixture was

incubated at 37°C for 60 min. The contents were centrifuged at 500 g for 5 min. 0.5 ml TCA was added to 0.8 ml of supernatant, the contents were vortexed and 0.5 ml thiobarbituric acid reagent was added. After mixing well, the mixture was heated in a boiling water bath for 30 min. The absorbance of the resulting pink colour was recorded at 532 nm. The hydroxyl radical generation was quantified in terms of MDA equivalents using a standard curve for MDA estimation.

Determination of nitrite

Nitrite was measured by Greiss reaction (29). The working Greiss reagent was freshly prepared by mixing equal volumes of NEDD and sulphanilamide (3.8 and 58 mM respectively made in 5 % phosphoric acid). 2×10^6 PMNL suspended in HBSS, pH 7.4 were pre-treated with 15 mM L-Arg for 10 minutes before stimulants were added to each reaction mixture. The final volume was made up to 1.0 ml with HBSS. The reaction mixture was incubated at 37°C for 60 min. in a shaking water bath and the contents were centrifuged at 500g for 5 min. at 4°C . 0.5 ml of supernatant was mixed with 0.5 ml of Greiss reagent and incubated for 15 minutes at 25°C . The absorbance was read at 550 nm against reagent blank. A standard curve was plotted with NaNO_2 in the concentration range of 0-2 mM, which was used to calculate the nitrite concentration in unknown samples.

RESULTS

API was isolated from 200 ml of human serum containing 12.52 g of total protein with 650 units of API activity. The first purification step, involving 50% ammonium sulphate precipitation in presence of DTT and fumed silica resulted in 9.8 fold purification. The specific activity increased almost 10 fold from 0.052 to 0.512, although recovery of total activity was only 52.3%. After the second step in which DEAE-Cellulose column chromatography was carried out (Fig.1), the specific activity increased to 0.7 with an overall purification of 13.4 fold. Thirty four fractions were pooled which had total API activity corresponding to 33.8 % of original. The pooled fractions gave a single band on SDS-PAGE indicating its homogeneity (Inset of Fig. 1). The optimal activator concentration, PMNL population and incubation time were worked out in preliminary experiments. It was found that optimal concentration of PMA, FMLP, OZ and AA for PMNL stimulation were 0.01 $\mu\text{g}/\text{ml}$, 4.3 $\mu\text{g}/\text{ml}$, 1.5 mg/ml and 15.2 $\mu\text{g}/\text{ml}$ respectively. A uniform population of PMNL (2×10^6 cells/ml) was used for all the activators and the optimum incubation time was found to be 30 min at 37°C .

The API activity remained unaltered on pre-incubation of API with resting PMNL. However when PMNL were activated with PMA, API activity was reduced to 76.7%. The decrease in API activity was 20%, 12% and 16.6%

Figure 1. Elution Profile of API on DEAE-Cellulose. A linear gradient formed by 0.01M phosphate buffer and 0.2 M NaCl, pH 6.0 was initiated at fraction No. 25 and 3.0 ml fractions were collected. Fractions from 47 to 80 were pooled and preserved for further studies. The inset shows the SDS PAGE pattern of molecular weight markers (lane 1), crude API preparation (Lane 2) and purified API (lane 4). The different molecular weight standards were A 20 KDa, B (45 KDa), C (66 KDa) and D (84 KDa) respectively.

Figure 2. Inactivation of API by stimulated PMNL with different activators, PMA (1), FMLP (2), OZ (3) and AA (4).

when PMNL were activated with FMLP, OZ and AA respectively (Fig. 2). The inclusion of scavengers of reactive oxygen species resulted in the improvement of EIA. SOD and catalase restored about 18.0% of EIA, whereas mannitol, a scavenger of hydroxyl radical and sodium azide, an inhibitor of myeloperoxidase restored 13.0% and 17.30% EIA respectively (Fig. 3). The pre-treatment of PMNL with L-Arg before activation by the four agonists protected API against inactivation. The protection offered by L-Arg varied with the type of activator used and was maximum with PMA (Fig 4). API activity was restored from 65% to 80.10% on activation of L-Arg pre-treated PMNL with PMA. The EIA was

Figure 3. Elastase inhibitory activity of API in absence and presence of ROS scavengers. The details are as follows; Neat API activity (A), with resting PMNL (B), with activated PMNL (C), with SOD and activated PMNL (D), with catalase and activated PMNL (E), with mannitol and activated PMNL (F) and with sodium azide and activated PMNL (G).

Figure 4 Effect of L-Arg on the elastase inhibitory activity of API in presence of PMNL activated with different agonists. The control (1) represents the EIA in presence of L-Arg only. 2, 3, 4 and 5 represent EIA in presence of agonists ; PMA, FMLP, OZ and AA with (●) and without (○) L-Arg respectively.

restored from 69.40% to 78.20%, from 66.40% to 79.30 % and from 66.20 % to 78.00% when the Arg pre-treated PMNL were activated with FMLP, zymosan and AA respectively (Fig. 4).

The superoxide generation was studied in freshly pooled PMNL preparations. The total superoxide production was 38.98, 15.44, 20.51 and 28.1 n moles/ 2×10^6 cells/10 min. for PMA, FMLP, OZ and AA respectively. L-Arg. reduced the superoxide production by 14.6%, 32.30%, 26.24% and 68.9% in presence of PMA, FMLP, OZ and AA respectively (Fig. 5). The total inhibition of superoxide production showed

Figure 5. Superoxide production by PMNLs (2×10^6 cells) in presence (●) and absence (○) of L-Arg. The PMNL were activated by PMA (1), FMLP (2), OZ (3) and AA (4) respectively.

Figure 6. Inhibition of superoxide production by different concentrations of L-Arg. 2×10^6 cells (PMNL) were pre-incubated with L-Arg (concentrations ranging from 2 to 14 mM) before activator PMA (10ng) was added in a final volume of 1.0ml HBSS, pH 7.4 containing 1.26 mg cytochrome C. Superoxide was measured by SOD-inhabitable cytochrome C reduction. Control represents superoxide production with out L-Arg.

concentration dependence with L-Arg and the maximum inhibition was observed at 14 mM L Arg under our experimental conditions (Fig. 6). PMA was the most potent inducer of hydroxyl radical generation by PMNL whereas FMLP had no response at all. Hydroxyl radical generation was found to be 114.96, 38.9 and 111.7 ng MDA equivalents of hydroxyl radical for PMA, OZ and AA respectively. As in case of

Figure 7. Hydroxyl radical generation in total absence (●) and presence (○) of 15 mM L-Arg. The activators used were PMA (1), OZ (2) and AA (3).

Figure 8. Nitrite production by PMNL stimulated with PMA (1), FMLP (2), OZ (3) and AA (4) in Presence (●) and absence (○) of L-Arg.

Figure 9. Effect of aminoguanidine on PMNL mediated inactivation of API.

1. EIA of neat API
2. EIA in presence of PMA activated PMNL.
3. EIA in presence of L-Arg pretreated and PMA activated PMNL
4. EIA in presence of aminoguanidine and L-Arg pre-treated, PMA activated PMNL.

superoxide, L-Arg reduced the generation of hydroxyl radicals. The extent of decrease was 33.98%, 22.16% and 40.46% for PMA, OZ and AA respectively (Fig. 7)

The generation of NO in presence of L-Arg was measured in terms of nitrite, since NO is unstable to measure. The L-Arg pretreated PMNLs released significant amounts of nitrite on stimulation with the agonists. The nitrite production was more pronounced by AA both in presence and absence of L-Arg (Fig. 8) as compared to other three activators. The effect of nitric oxide synthase inhibitor, aminoguanidine on API inactivation was also studied. Aminoguanidine decreased the nitrite production by 81% from 1.12 to 0.21 mM in presence of PMA as a stimulant. The protection offered to API against inactivation by L Arg was reversed in presence of aminoguanidine and the API lost 21% EIA (Fig. 9).

DISCUSSION

In resting PMNLs, the NADPH oxidase is dormant with unassembled subunits located in the cytosol and plasma membrane which gets activated on selective stimuli (1,30,31,32). All the four agonists used by us inactivated API molecule, though the maximum inactivation was achieved with PMA followed by FMLP, AA and OZ in that order. It is well recognized that more than one signaling pathway can activate the same response in neutrophils (33). PMA (a non physiological stimulus) activates cells by stimulating extracellular signal regulated kinases (ERKs) through a PKC dependent pathway, whereas OZ involves the tyrosine kinase, Pyk2 dependent activation of cytosolic phospholipase A2 and the stimulation is further mediated by FCγ receptor and not by complement receptor C3b protein (2,3,34). The precise mechanism for AA mediated activation of NADPH oxidase is still elusive, however several studies suggest that AA induces structural changes in NADPH oxidase components that may promote productive interactions between different oxidase subunits to provide a fully active oxidase or directly affecting the function of flavocytochrome b (35,36,37). FMLP, a chemoattractant bacterial peptide, is a potent activator of neutrophil degranulation (38) and the activation occurs via diverse pathways including phosphorylation/inactivation of the FOXO subfamily of fork-head transcription factors (FKHR, FKHR-L1 and AFX) through the phosphatidylinositol-3-kinase/Akt (protein kinase B) and the RAS mitogen-activated protein kinase pathways (39,40,41,42).

SOD, a selective scavenger of superoxide and catalase, which decomposes H_2O_2 , protected the API against oxidative inactivation to some extent. Mannitol, a scavenger of hydroxyl radical and sodium azide also offered protection to API molecule to appreciable and varying extents. These observations demonstrate the

multifactorial nature of inactivation process. It seems that O_2^- , H_2O_2 , $^{\bullet}OH$ and MPO dependent ROS are responsible for API inactivation. The role of O_2^- is pivotal, since it is the precursor of almost all other pro-oxidant species. Hydroxyl radical is believed to be the most lethal species and is formed either spontaneously from H_2O_2 or by metal catalyzed oxidation of H_2O_2 (43,44). The lactoferrin released by secondary granules may provide the catalytic iron (as a result of damage by different ROS) which may be responsible for metal dependent generation of hydroxyl radical (45). The pH inside the phagocyte vacuoles of PMNL has been reported to be acidic which is quite conducive for iron release from the lactoferrin. Hydroxyl radical might also be directly generated by the decomposition of peroxynitrite (16).

All the four activators caused a significant increase in nitrite content in PMNL pre-incubated with L-Arg. Arachidonic acid gave the maximum stimulus to PMNL for nitrite generation. Without the L-Arg pretreatment, nitrite generation could not be observed after the activation of PMNL. The pre-treatment of PMNL with L-Arg caused appreciable decrease in the superoxide and hydroxyl radical generation, the former being L-Arg concentration dependent. In addition to the mediating endothelial relaxation of vascular smooth muscle (46), inhibiting platelet aggregation and adhesion and mediating tissue damage (47, 48) nitric oxide has also been found to modulate superoxide radical generation (11, 16, 17, 18, 49). In our studies inclusion of L-Arg. offered protection against PMNL mediated oxidative inactivation, irrespective of the type of activator used. The NOS inhibitor, aminoguanidine suppressed the nitrite release and there was no protection to API inactivation thereby indicating that NO scavenges superoxide so that the later is not available to inactivate API *per se* or indirectly through the generation of other ROS. The other inhibitors of NOS like L-NMMA and L-NAME were not used because of their non-specificity (50)

Our results strongly suggest that O_2^- is scavenged by NO in such a way that oxidative damage to the target protein (API) is minimized. This is contrary to what should be expected after the generation of peroxynitrite from O_2^- and NO (11, 15). Possibly NO is metabolized very fast because of its short half life of 1-5 sec. (11) and converted to less lethal species like nitrite and nitrate. Earlier, it was believed that nitric oxide inhibits O_2^- production at NADPH-oxidase level (13). Contrary to this, our results strongly suggest that nitric oxide scavenges the O_2^- directly rather than inhibiting the NADPH oxidase. The observation that nitrite release substantially decreased on inclusion of the SOD (which scavenges the superoxide ion) along with L-Arg gives enough credence to the belief that nitric oxide under our experimental conditions, directly

scavenges the superoxide radical, resulting in the formation of less toxic species like nitrite which protect the API from oxidative inactivation.

In conclusion, this study on one hand points to the multifactorial nature of oxidative inactivation of API by activated PMNLs *in vitro* which is reversed by nitric oxide and on the other hand suggests that NO scavenges superoxide directly rather than inhibiting NADPH oxidase.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the facilities provided by Director, SKIMS, Srinagar, proofreading of the manuscript by Dr Nusrat Hussain and the technical assistance by Mr. Mushtaq A Malik and B A Pathan.

REFERENCES

1. Malech, H. L., Gallin, M. D. (1987) Neutrophils in human disease. *N. Engl. J. Med.* 317,687-694
2. Geiszt, M. and Leto, T. L. (2004) The Nox family of NAD(P)H oxidase : host defence and beyond. *J. Biol. Chem.* 279, 51715-51718.
3. Quinn, M.T. and Gauss, K. A. (2004) Structure and regulation of the neutrophil respiratory burst: comparison with non-phagocyte oxidases. *J. Leukoc. Biol.* 76,760-781.
4. Smith, J. A. (1994) Neutrophils, host defense and inflammation: a double-edged sword. *J. Leukoc. Biol.* 56, 672-686.
5. Sethi, S., Sing, M. P. and Dikshit, M. (1999) Nitric oxide- mediated augmentation of polymorphonuclear free radical generation after hypoxia-reoxygenation. *Blood* 93, 333-340.
6. Carreras, M. C., Pargament, G. A., Catz, S. D., Poderoso, J. J. and Boveris, A. (1994) Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. *FEBS Lett.* 341, 65-71.
7. Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) Oxidative chemistry of peroxynitrite. *Methods Enzymol.* 233, 229-240.
8. Moreno-Manzano, V., Ishikawa, Y. and Lucio-Cazana J. (2000) Selective involvement of superoxide anion, but not downstream compounds hydrogen peroxide and peroxynitrite, in tumour necrosis factor- α -induced apoptosis of rat mesangial cells. *J. Biol. Chem.* 275, 12684-12691.
9. Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997) Oxidants as stimulators of signal transduction. *Free Radic. Biol. Med.* 22, 269-285.
10. Rabadji, R. C., Brigagao, M. R. P. L., Camerero, V. C. P. C. and Colepicolo, P. (1996) Oscillation of reactive oxygen species released by activated neutrophils. *Biological Rhythm Research* 27, 269-280.
11. Salvemini, D., Ischiropoulos, H. and Cuzzocrea, S. (2003) Roles of nitric oxide and superoxide in inflammation. *Methods Mol. Biol.* 225, 291-303.
12. Ricciardolo, F.L.M. (2003) Multiple roles of nitric oxide in the airways. *Thorax* 58, 175-182.
13. Clancy, R.M., Leszczynska-Piziak, J. and Abramson, S.B. (1992) Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.* 90, 1116-1122.
14. Beckman, J.S. and Crow, J.P. (1993) Pathological importance of nitric oxide, superoxide and peroxynitrite formation. *Biochem. Soc. Trans.* 21, 330-334.
15. Rodenas, J., Mitjavila, M. T. and Carbonell, T. (1995) Simultaneous generation of nitric oxide and superoxide by inflammatory cells in rats. *Free Radic. Biol. Med.* 18, 869-875.
16. Ross, D., Indranil, D., Nathan, J. and Yedy, I. (1998) Effect of antithyroid drugs on hydroxyl radical formation and α -1-proteinase inhibitor inactivation by neutrophils : therapeutic implications. *J. Pharm. Exp. Ther.* 285,1233-1238.
17. Brune, B. and Lapetine, E. G. (1989) Activation of cytosolic ADP-ribosyl transferase by nitric oxide generating agents. *J Biol Chem* 269, 8455-8461.
18. Piper, G. M., Clarke, G. A. and Gross, G. J. (1994) Stimulatory and inhibitory action of nitric oxide donor agents vs nitrovasodilators on reactive oxygen production by isolated polymorphonuclear leukocytes. *J Pharmacol. Exp. Ther.* 269, 451.
19. Morikawa, M., Inoue, M., Tokumaru, S. and Kogo, H. (1995) Enhancing and inhibitory effects of nitric oxide on superoxide anion generation in human polymorphonuclear leukocytes. *Br J Pharmacol* 115, 1303.
20. Pryor, W. A., Dooley, M M. and Church, D. F. (1984) Inactivation of human α -1-proteinase inhibitor by gas phase cigarette smoke. *Biochim. Biophys. Res. Comm.* 122,676-681.
21. Fagerhol, M. K. and Cox, D. W. (1981) The Pi polymorphism: Genetic, biochemical and clinical

- aspects of human α -1- antitrypsin. *Adv. Human. Genet.* 11, 1-10.
22. Travis, J. and Salvensen, G. S. (1983) Human plasma proteinase inhibitors. *Ann. Rev. Biochem.* 52, 655-709.
23. Beatty, K., Beith, J. and Travis, J. (1980) Kinetics of association of serine proteinases with native and oxidized α -1-proteinase inhibitor and α -1-antichymotrypsin. *J. Biol. Chem.* 255: 3931-3935.
24. Arthur, B. Z. (1993) Genetic disease of the pulmonary parenchyma, In: *Pulmonary disease (fifth ed.)*. Eds. Gerald L Baum and Emanuel Wolinsky, Boston: Little, Brown and company, pp1785-1813.
25. Khan, A.R., Mir, M. M., Wani, J. I. Siddiqi, M.A. and Salahuddin, M. (1998) Superoxide generation by resting and activated polymorphonuclear leucocytes obtained from smokers and non-smokers. *Med. Sci. Res.* 26, 471-473.
26. Glaser, C.B., Chamorro, M., Crowley, R., Karic, L., Childs, A. and Calderon, M. (1982) The isolation of α -1- proteinase inhibitor by a unique procedure designed for industrial application. *Anal. Biochem.* 124, 364-371.
27. Beatty, K., Beith, J. and Travis, J. (1982) Determination of oxidized alpha-1-proteinase inhibitor. *J. Lab. Clin. Med.* 100, 86-92.
28. Greenwald, R. A., Scott, W. Rush, S. A. M. and Zee, V.W. (1989) Conversion of superoxide generated by polymorphonuclear leucocytes to hydroxyl radical : A direct spectrophotometric detection system based on degradation of deoxyribose. *Free. Radic. Biol. Med.* 6, 385-392.
29. Bennett, B.M., Kobus, S.M., Brien, J.F., Nakatsu, K. and Marks, G.S. (1986) Requirement of reduced, unliganded hemoproteins for the hemoglobin and myoglobin mediated biotransformation of glyceryl trinitrate. *J. Pharmacol. Exp. Ther.* 237, 629-632.
30. Babior, B. M., Lambeth, J. D. and Nauseef, W. (2002). The neutrophil NADPH oxidase. *Arch. Biochem. Biophys.* 397, 342-344.
31. Babior, B. M. (1999) NADPH oxidase: an update. *Blood* 93, 1464-1476.
32. Leto, T. L., Garrett, M. C., Fujii, H. and Nunoi, H. (1991) Characterization of neutrophil NADPH oxidase factor p47phox and p67phox from recombinant baculoviruses. *J. Biol. Chem.* 266, 19812-19818.
33. Hazan, I., Dana, R., Granot, Y. and Levy, R. (1997) Cytosolic phospholipase A2 and its mode of activation in human neutrophils by opsonized zymosan, correlation between 42/44 kDa mitogen-activated kinase, cytosolic phospholipase A2 and NADPH oxidase. *Biochem. J.* 326, 867-876.
34. Hazan, I., Seger, R. and Levy, R. (2000) The requirement of both extracellular regulated kinase and p38 mitogen-activated protein kinase for stimulation of cytosolic phospholipase A₂ activity by either Fc γ RII A or Fc α RIIIB in human neutrophils. *J. Biol. Chem.* 275, 12416-12423.
35. Shmelzer, Z., Haddad, N., Admon, E., Pessachh, I., Leto, T. L., Eitan-Hazan, Z., Hershinkel, M. and Levy, R. (2003) Unique targeting of cytosolic phospholipase A2 to plasma membranes mediated by the NADPH oxidase in phagocytes. *J. Cell Biol.* 162, 683-692.
36. Foubert, T. R., Burritt, J. B., Taylor, R. M. and Jesaitis, A. J. (2000) Structural changes are induced in human neutrophil cytochrome b by NADPH oxidase activators, LDS, SDS, and arachidonate: intermolecular resonance energy transfer between trisulfopyrenyl-wheat germ agglutinin and cytochrome b558. *Biochim. Biophys. Acta.* 1557, 321-231.
37. Ueno, N., Murakami, M., Tanioka, T., Fujimori, K., Tanabe, T., Urade, Y. and Kudo, I. (2001) Coupling between cyclooxygenase, terminal prostanoid synthase, and phospholipase A2. *J. Biol. Chem.* 276, 34918-34927
38. Tuluc, F., Garcia, A., Bredeteau, O., Meshki, J. and Kunapuli S.P. (2004) Primary granule release from human neutrophils is potentiated by soluble fibrinogen through a mechanism depending on multiple intracellular signaling pathways. *Am. J. Physiol. Cell Physiol.* 287, 1264-1272.
39. Crossley, L.J. (2003) Neutrophil activation by fMLP regulates FOXO (forkhead) transcription factors by multiple pathways, one of which includes the binding of FOXO to the survival factor Mcl-1. *J. Leukoc. Biol.* 74, 583-592.
40. Jacob, C., Szilagyi, C., Allen, J.M., Bertrand, C. and Lagente, V. (2004) Role of PDE4 in superoxide anion generation through p44/42 MAPK regulation: a cAMP and a PKA-independent mechanism. *Br. J. Pharmacol.* 143, 257-268.
41. Walters, J.D. and Nakkula, R.J. (2003) Ciprofloxacin transport by chemoattractant-activated polymorphonuclear leukocytes: regulation by priming and protein kinase C. *Antimicrob. Agents Chemother.* 47, 3345-3348.

42. Fujita, T., Zawawi, K.H., Kurihara, H. and Van Dyke, T.E. (2005) CD38 cleavage in fMLP- and IL-8-induced chemotaxis is dependent on p38 MAP kinase but independent of p44/42 MAP kinase. *Cell Signal.* 17, 167-75. (Medline).
43. Halliwell, B., Gutteridge, J.M.C. and Blake, D.R. (1985) Metal ion and oxygen radical reaction in human inflammatory joint disease. *Phil. Trans. R. Soc.* 311, 659-671.
44. Aruoma, O.I., Halliwell, B. and Dizdaroglu, M. (1991) Copper ion dependent damage to the bases in DNA in presence of hydrogen peroxide. *Biochem. J.* 273, 601-604.
45. Ambruso, D.R. and Johnson Jr., R.B. (1981) Lactoferrin enhances hydroxyl radical production by human neutrophil, neutrophil particulate fractions and an enzyme generating system. *J. Clin. Invest.* 67, 352-360.
46. Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelial derived relaxing factor. *Nature* 327; 524-527.
47. Kubes, P., Suzuki, M. and Granger, D.N. (1991), Nitric oxide: an endogenous modulator of leucocyte adhesion. *Proc. Natl. Acad. Sci. (USA)* 88, 4651-4655.
48. Mulligan, M.S., Hevel, J.M., Marletta, M. A. and Ward, P.A. (1991) Tissue injury caused by deposition of immune complexes is L-Arg dependent. *Proc. Natl. Acad. Sci. (USA)* 88, 6338-6341.
49. Rubanyi, G. M., Ho, E. H., Cantor, E. H., Lumma, W.C. and Botelhi, L. H. P. (1991) Cytoprotective function of nitric oxide. Inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* 181, 1392-1395.
50. Peterson, D.A. (1992) The non-specificity of specific nitric oxide synthase inhibitors. *Biochem. Biophys. Res. Commun.* 187, 797-800.