THE EFFECTS OF THE *Pseudomonas aeruginosa*-DERIVED PIGMENT, 1-HYDROXYPHENAZINE, ON CALCIUM METABOLISM AND RELEASE OF PRIMARY GRANULE ENZYMES FROM ACTIVATED HUMAN NEUTROPHILS *IN VITRO*

by

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DEDICATED TO MY MOTHER, TEBOGO

SUMMARY

The effects of pathologically-relevant concentrations (0.38-12.5μM) of the pro-inflammatory, *Pseudomonas aeruginosa*-derived pigment, 1-hydroxyphenazine (1-HP), on Ca^{2+} metabolism and intracellular cyclic AMP in FMLP (1μM)-activated human neutrophils, as well as on the release of myeloperoxidase (MPO) and elastase from these cells, have been investigated *in vitro*. Ca^{2+} fluxes were measured using the combination of a fura-2/AM-based spectrofluorimetric method and radiometric procedures which together enabled the distinction between the net efflux and influx of the cation, while colorimetric and radioimmunoassay methods were used to measure granule enzymes and cAMP respectively.

Coincubation of neutrophils with 1-HP did not affect intracellular cAMP or FMLP-activated release of Ca^{2+} from intracellular stores, but did retard the subsequent decline in the concentration of cytosolic free calcium. These effects of 1-HP on the clearance of Ca^{2+} from the cytosol of activated neutrophils were associated with inhibition of efflux of the cation, probably as a result of the antagonistic effect of 1-HP on the plasma membrane Ca^{2+}-ATPase and are the probable cause of increased release of MPO and elastase from the cells.

Because I was unaware of any pharmacologic strategies to potentiate the activity of the plasma membrane Ca^{2+}-ATPase, thereby overcoming the inhibitory effects of 1-HP on the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils, I reasoned that pharmacologic enhancement of the cAMP-regulated endo-membrane Ca^{2+}-ATPase, which sequesters/resequesters the cation into intracellular stores, may offer an alternative strategy to restore Ca^{2+} homeostasis in 1-HP-treated neutrophils. Treatment of neutrophils with intracellular cAMP-elevating agents such as dibutyryl cAMP (cell-permeable analogue), rolipram and GR61170X (selective inhibitors of type 4 phosphodiesterase) and CGS21680 and IB-MECA (selective adenosine type 2a and 3 receptor agonists respectively), and to a much lesser extent with theophylline (non-selective phosphodiesterase inhibitor) neutralized the enhancing effects of 1-HP on elastase release by stimulated neutrophils. However, the β_2-adrenoreceptor agonists, salbutamol and salmeterol, were ineffective, which

correlated well with the inability of these agents to elevate cAMP in neutrophils.

Dibutyryl cAMP and rolipram were also investigated for their effects on 1-HP-mediated inhibition of the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils. Both cAMP-elevating agents neutralized the inhibitory effects of the pigment on removal of Ca^{2+} from the cytosol, resulting in restoration of Ca^{2+} homeostasis to the cells. These observations strengthen the proposed mechanistic link between 1-HP-mediated inhibition of the clearance of cytosolic Ca^{2+} and enhancement of degranulation in stimulated neutrophils.

In conclusion, the *Pseudomonas aeruginosa*-derived pigment, 1-HP, possesses potentially harmful pro-inflammatory properties as a result of interference with the handling of Ca^{2+} by stimulated neutrophils. cAMP-elevating agents, particularly selective type 4 phosphodiesterase inhibitors and adenosine receptor agonists operative at the level of type A2a receptors may be potentially useful in patients colonized by *Pseudomonas aeruginosa* since they antagonize the pro-inflammatory activity of 1-HP. These anti-inflammatory effects of the cAMP-elevating agents are achieved indirectly by increasing the efficiency of the endo-membrane Ca^{2+}-ATPase, which compensates for, and bypasses 1-HP-mediated inhibition of the plasma membrane Ca^{2+} extrusion system.

Key words: Neutrophils, *Pseudomonas aeruginosa*, 1-hydroxyphenazine (1-HP), calcium, cyclic AMP, Dibutyryl cAMP, phosphodiesterase inhibitor, adenosine receptor agonists, rolipram, CGS21680.

SAMEVATTING

Die uitwerking van die patologies-verwante konsentrasies (0.38-12.5μM) van die pro-inflammatoriese, *Pseudomonas aeruginosa*-afkomstige pigment, 1-hidroksiefenasien (1-HP), op die Ca^{2+} metabolisme en intrasellulêre sikliese AMP in FMLP (1μM)-geaktiveerde mensneutrofiele, sowel as op die vrystelling van miëloperoksidase (MPO) en elastase deur hierdie selle, is *in vitro* ondersoek. Ca^{2+} flukse is bepaal deur gebruik te maak van 'n kombinasie van fura-2 AM-gebaseerde spektrofluorimetriese- en radiometriese metodes wat saam die onderskeid tussen die totale effluks en influks van die katioon kon tref, terwyl kolorimetriese en radio-immuunbepalingsmetodes gebruik is om granule-ensieme en cAMP, respektiewelik, te bepaal.

Die behandeling van neutrofiele met 1-HP het geen effek op die intrasellulêre cAMP of FMLP-geaktiveerde vrystelling van Ca^{2+} vanuit die intrasellulêre store gehad nie, maar het die daaropvolgende daling in die konsentrasie van sitosoliese vry kalsium, vertraag. Hierdie uitwerking van 1-HP op die verwydering van Ca^{2+} uit die sitosol van geaktiveerde neutrofiele, het met die inhibisie van die effluks van die katioon gepaardgegaan en kan moontlik toegeskryf word aan die antagonistiese uitwerking van 1-HP op die plasmamembraan Ca^{2+}-ATPase en is ook moontlik die oorsaak van die verhoogde vrystelling van MPO en elastase deur die selle.

Aangesien ek onbewus was van enige farmakologiese strategieë wat aangewend kon word om die aktiwiteit van die plasmamembraan Ca^{2+}-ATPase te verhoog om sodoende die inhiberende uitwerking van 1-HP op die verwydering van Ca^{2+} uit die sitosol van FMLP-geaktiveerde neutrofiele te oorkom, het ek geredeneer dat die farmakologiese verhoging van die cAMP-gereguleerde endomembraan-Ca^{2+}-ATPase, wat die katioon in die intrasellulêre store sekwestreer of hersekwestreer, 'n alternatiewe strategie mag bied om die Ca^{2+} homeostase te herstel in 1-HP-behandelde neutrofiele. Behandeling van neutrofiele met middels wat intrasellulêre cAMP verhoog bv. dibutiriel cAMP (seldeurlaatbare analoog), rolipram en GR61170X (selektiewe inhibeerders van tipe 4 fosfodiesterase), CGS21680 en IB-MECA (selektiewe adenosien tipe 2a en 3 reseptor agoniste respektiewelik), en teofillien (nie-selektiewe fosfodiesterase inhibeerder), het

die verhogende uitwerking van 1-HP op elastase vrystelling deur gestimuleerde neutrofiele, geneutraliseer, hoewel die effek met teofillien aansienlik kleiner was. Die beta2-adrenoseptor agoniste, salbutamol en salmeterol, was egter oneffektief, wat goed met die onvermoë van hierdie agente om cAMP in neutrofiele te verhoog, gekorreleer het.

Die uitwerking van dibutiriel cAMP en rolipram op die 1-HP-bemiddelde inhibisie van die verwydering van Ca^{2+} uit die sitosol van FMLP-geaktiveerde neutrofiele, is ook ondersoek. Beide cAMP-verhogende middels het die effekte van die pigment op die opruiming van Ca^{2+} vanuit die sitosol, geneutraliseer, wat tot die herstel van die Ca^{2+}-homeostase in die selle gelei het. Hierdie waarnemings versterk die voorgestelde meganistiese skakel tussen die 1-HP bemiddelde inhibisie van die opruiming van sitosoliese Ca^{2+} en die verhoging van degranulasie in gestimuleerde neutrofiele.

Ten slotte, die *Pseudomonas aeruginosa*-afkomstige pigment, 1-HP, besit potensieel skadelike pro-inflammatoriese eienskappe as gevolg van die belemmerende uitwerking daarvan op die hantering van Ca^{2+} deur gestimuleerde neutrofiele. cAMP-verhogende agente, veral die selektiewe tipe 4 fosfodiesterase inhibeerders en adenosien reseptor agoniste werksaam op die vlak van tipe A2a reseptore, mag potensieel bruikbaar wees in pasiënte wat deur *Pseudomonas aeruginosa* gekoloniseer is, aangesien dit die pro-inflammatoriese aktiwiteit van 1-HP, teenwerk. Die anti-inflammatoriese uitwerking van die cAMP-verhogende agente word indirek bereik deur die doeltreffendheid van die endomembraan Ca^{2+}-ATPase te verhoog, wat kompenseer vir die plasmamembraan Ca^{2+} verwyderingsisteem en die belemmerende uitwerking van 1-HP daarop.

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ATP	--> Adenosine triphosphate
BAL	--> Bronchoalveolar lavage
Ca^{2+}	--> Calcium ion
$^{45}Ca^{2+}$	--> Calcium-45 chloride
CaBP	--> Calcium binding protein
cAMP	--> Adenosine 3', 5'-monophosphate
CBF	--> Ciliary beat frequency
cDNA	--> Copy deoxyribonucleic acid
CF	--> Cystic Fibrosis
CFTR	--> Cystic Fibrosis Transmembrane Conductance regulator
Cl^-	--> Chloride
CPA	--> Cyclopentyladenosine
DAG	-->Diacylglycerol
DNA	--> Deoxyribonucleic acid
ELF	--> Epithelial lining fluid
FMLP	--> N-formyl-methyl-leucyl-phenylalanine
FMLP/CB	--> N-formyl-methyl-leucyl-phenylalanine/cytochalasin-B
FURA-2/AM	--> 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5-'-methylphenoxy)-ethane-N,N,N',N'-tetra-acetic pentaacetoxy methylester oil
GSH	--> Glutathione
H_2O	--> Water
H_2O_2	--> Hydrogen peroxide
HLMC	--> Human lung mast cell
1-HP	-->1-Hydroxyphenazine
HOCl	--> Hypochlorous acid
IgA	--> Immunoglobulin A
IgE	--> Immunoglobulin E
IgG	--> Immunoglobulin G
IL	--> Interleukin

IP_3	--> Inositol triphosphate
LPS	--> Lipopolysaccharide
MPO	--> Myeloperoxidase
mRNA	--> Messenger ribonucleic acid
Na^+	--> Sodium ion
NaCl	--> Sodium Chloride
NADPH	--> Nicotinamide-adenine-dinucleotide-phosphate-hydrogenase
NE	--> Neutrophil elastase
NO	--> Nitric Oxide
O_2^-	--> Superoxide
PAF	--> Platelet-activating-factor
PDE	--> Phosphodiesterase
PE	--> *Pseudomonas aeruginosa* elastase
PIP_2	--> Phosphatidylinositol-4,5-biphosphate
PKC	--> Protein kinace C
PLC	--> Phospholipase C
PMA	--> Phorbol-12-myristate acetate
Pyo	--> Pyocyanine
R	--> Receptor
SCID	--> Severe combined immunodeficiency
SLPI	--> Secretory leukoprotease inhibitor
SOD	--> Superoxide dimutase
TNFα	--> Tumor necrosis factor alpha
W^+	--> Wild type