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Effects of Adenosine Receptor Agonists of the A_1, A_{2A} and A_3 Subtypes on the Proinflammatory Activity of Human Neutrophils *In Vitro*

by

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Declaration

To my knowledge the work contained in this thesis is original, was undertaken by myself with occasional assistance as indicated in the acknowledgements. The interpretation and analysis of data were my primary responsibilities.

It is being submitted for the degree of Doctor of Philosophy at the University of Pretoria. It has not been submitted before for any degree or examination at any other university.

Signed: 

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Publications

Part of this thesis have been published in the following papers:

1. Visser SS, Theron AJ, Ramafi G, Ker JA & Anderson R. Apparent involvement of the A_{2A} subtype adenosine receptor in the anti-inflammatory interactions of CGS 21680, cyclopentyladenosine, and IB-MECA with human neutrophils. Biochemical Pharmacology 2000;60:993-999.

2. Anderson R, Visser SS, Ramafi G & Theron AJ. Accelerated resequestration of cytosolic calcium and suppression of the pro-inflammatory activities of human neutrophils by CGS 21680 *in vitro*. British Journal of Pharmacology 2000;130:717-724.

Summary

The apparent insensitivity of neutrophils to the anti-inflammatory effects of corticosteroids underscores the requirement for identifying agents which suppress neutrophil-driven inflammation. In a preliminary study, I was unable to demonstrate an inhibitory effect of glucocorticoids (dexamethasone) on the rapidly-activatable pro-inflammatory functions (superoxide production and elastase release) of human neutrophils activated with FMLP.

My subsequent research was directed at identifying the adenosine receptor (AR) subtypes which down-regulate the pro-inflammatory activities of human neutrophils, as well as the involvement of adenosine 3′,5′-cyclic monophosphate (cAMP) and its relationship to cellular handling of Ca^{2+} in mediating these effects. Neutrophils were treated with varying concentrations (0.01 – 1 μM) of AR agonists operative at A_1 (N^6-cyclopentyladenosine, CPA), A_{2A} (2(4-[(2-carboxyethyl)phenyl]ethylamino)-5′-N-ethylcarboxamidoadenosine, CGS 21680), and A_3 (N^6-(3-iodobenzyl-5′-N-methylcarbamoyladenosine, IB-MECA) receptors, after which they were activated with the chemoattractant , N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM). Intracellular cAMP, superoxide production, and elastase release were assayed using radioimmunoassay, lucigenin-enhanced chemiluminescence (LECL), and colorimetric procedures, respectively, while changes in the concentrations of cytosolic Ca^{2+} were monitored by fura-2-based spectrofluorimetry. CGS 21680, at all concentrations tested, inhibited superoxide production in a dose-related manner, while CPA and IB-MECA were effective only at the highest concentrations tested (0.5 – 1 μM). The release of elastase from activated neutrophils was also inhibited by all three AR agonists, but was more sensitive to CGS 21680 and IB-MECA than was superoxide production. The inhibitory effects of all 3 agonists on superoxide production and elastase release were associated with accelerated clearance of Ca^{2+} from the cytosol of activated neutrophils, and were effectively neutralized by pretreatment of the cells with the highly selective A_{2A}R antagonist, ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5yl amino]ethyl)phenol). Increased cAMP was detected in neutrophils treated with CGS 21680 and IB-MECA (1 μM). These data support the involvement of the A_{2A}R subtype in the suppression

of superoxide production and degranulation by activated human neutrophils, probably by cAMP-mediated alterations in Ca^{2+} handling.

The involvement of the $A_{2A}R$ subtype in regulating the pro-inflammatory activities of neutrophils as well as the biochemical mechanism thereof, were subsequently investigated. Cytosolic Ca^{2+} fluxes, measured by fura-2 spectrofluorimetry in combination with radiometric procedures which distinguish between net efflux and influx of the cation, in FMLP-activated neutrophils in the presence and absence of CGS 21680, were determined. Treatment of neutrophils with CGS 21680 did not affect the FMLP-activated release of Ca^{2+} from intracellular stores, but resulted in dose-related acceleration of the rate of decline in fura-2 fluorescence, as well as decreases in both efflux and store-operated influx of Ca^{2+} , compatible with enhancement of re-sequestration of the cation by the endo-membrane Ca^{2+}-ATPase. These effects on neutrophil Ca^{2+} handling were associated with increased intracellular cyclic AMP and with inhibition of oxidant production and release of elastase. In contrast, treatment of neutrophils with ZM 241385 (2.5 μM), prevented the transient increase in cyclic AMP in FMLP-activated neutrophils which was associated with delayed sequestration of incoming Ca^{2+} during store-operated influx. The CGS 21680-mediated reduction of Ca^{2+} efflux from FMLP-activated neutrophils was also antagonized by pretreatment of the cells with ZM 241385 (2.5 μM), as well as by thapsigargin (1 μM), an inhibitor of the endo-membrane Ca^{2+}-ATPase. ZM 241385 also neutralized the cyclic AMP-elevating and anti-inflammatory interactions of CGS 21680 with neutrophils.

In conclusion, my results are compatible with a role for the $A_{2A}R$ in regulating the pro-inflammatory activities of human neutrophils by promoting cyclic AMP-dependent sequestration of cytosolic Ca^{2+}.

Keywords:
Adenosine; A_{2A} receptors; calcium; calcium influx; calcium efflux; CGS 21680; CPA; cyclic AMP; dexamethasone; elastase; IB-MECA; neutrophils; superoxide; ZM 241385.

Samevatting

Die klaarblyklike ongevoeligheid van neutrofiele vir die anti-inflammatoriese effekte van kortikosteroide beklemtoon die behoefte om agense te identifiseer wat neutrofiel-gedrewe inflammasie onderdruk. Ek was nie in staat om in 'n voorafgaande studie 'n onderdrukkende effek van glukokortikoide op die snel-aktiveerbare pro-inflammatoriese funksies (superoksied produksie en elastase vrystelling) van menslike neutrofiele wat geaktiveer was met FMLP, aan te toon nie.

My daaropvolgende navorsing was gemik op identifisering van die adenosien reseptor (AR) subtipes wat die pro-inflammatoriese aktiwiteite van menslike neutrofiele af-reguleer, asook die betrokkenheid van adenosien 3′,5′-sikliese monofosfaat (sAMF) en sy verhouding tot sellulêre hantering van Ca^{2+} in die bemiddeling van hierdie effekte. Neutrofiele is behandel met verskillende konsentrasies (0.01 – 1 μM) van AR agoniste wat werksaam is op A_1 (N^6-cyclopentyladenosine, CPA), A_{2A} (2(4-[(2-carboxyethyl) phenyl]ethylamino)-5′-N-ethylcarboxamidoadenosine, CGS 21680), en A_3 (N^6-93-iodobenzyl-5′-N-methylcarbamoyladenosine, IB-MECA) reseptore, waarna hulle aktiveer is met die chemolokmiddel, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM). Intrasellulêre sAMF, superoksied produksie, en elastase vrystelling is bepaal met radio-immuunbepaling, lucigenin-versterkte chemiluminessensie (LECL), en kolorimetriese prosedures, respektiewelik, terwyl veranderinge in die konsentrasies van sitosoliese Ca^{2+} moniteer is met fura-2-baseerde spektrofluorimetrie. CGS 21680, het by alle konsentrasies getoets, superoksied produksie inhibeer op 'n dosis-afhanklike wyse, terwyl CPA en IB-MECA slegs effektief was in die hoogste konsentrasies getoets (0.5-1 μM). Die vrystelling van elastase vanaf geaktiveerde neutrofiele is ook inhibeer deur al drie AR agoniste, maar was meer gevoelig vir CGS 21680 en IB-MECA as wat superoksied produksie was. Die inhibitoriese effekte van al drie agoniste op superoksied produksie en elastase vrystelling het gepaard gegaan met versnelde opruiming van Ca^{2+} vanaf die sitosol van geaktiveerde neutrofiele, en was doeltreffend neutraliseer deur vooraf behandeling van die selle met die hoogs selektiewe A_{2A} antagonis, ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo [2,3-*a*] [1,3,5] triazin-5yl amino] ethyl) phenol). Verhoogde sAMF is gevind in

neutrofiele wat behandel was met CGS 21680 en IB-MECA (1 μM). Hierdie bevindings ondersteun die betrokkenheid van die $A_{2A}R$ subtipe in die onderdrukking van superoksied produksie en degranulasie van geaktiveerde neutrofiele, waarskynlik via sAMF-bemiddelde veranderinge in Ca^{2+} hantering.

Die betrokkenheid van die $A_{2A}R$ subtipe in die regulering van die pro-inflammatoriese aktiwiteite van neutrofiele asook die biochemiese meganisme daarvan, is vervolgens ondersoek. Sitosoliese Ca^{2+} vloeiings, bepaal met fura-2 spektrofluorimetrie in kombinasie met radiometriese bepalings wat onderskei tussen netto uitvloeiing en invloeiing van die katioon, is bepaal in FMLP-geaktiveerde neutrofiele in die teenwoordigheid en afwesigheid van CGS 21680. Behandeling van neutrofiele met CGS 21680 het nie die FMLP-geaktiveerde vrystelling van Ca^{2+} vanaf intrasellulêre store beïnvloed nie, maar het gelei tot dosis-verwante versnelling van die tempo van afname in fura-2 fluoressensie, asook afname in beide uitvloeiing en stoor-beheerde invloeiing van Ca^{2+}, verenigbaar met versterking van hersekwestrasie van die katioon deur die endomembraan Ca^{2+}-ATPase. Hierdie effekte op neutrofiel Ca^{2+} hantering het gepaard gegaan met verhoogde intrasellulêre sikliese AMF en met inhibisie van oksidant produksie en vrystelling van elastase. In teenstelling, behandeling van neutrofiele met die selektiewe $A_{2A}R$ antagonis, ZM 241385 (2.5 μM), het die verbygaande styging in sikliese AMF in FMLP-geaktiveerde neutrofiele voorkom en het gepaard gegaan met vertraagde sekwestrasie van inkomende Ca^{2+} tydens stoor-beheerde invloeiing. Die CGS 21680-bemiddelde vermindering van Ca^{2+} uitvloeiing vanaf FMLP-geaktiveerde neutrofiele is ook teengewerk deur vooraf-behandeling van die selle met ZM 214385 (2.5 μM), asook deur thapsigargin (1 μM), 'n inhbitor van die endomembraan Ca^{2+}-ATPase. ZM 241385 het ook die sikliese AMF-verhogende en anti-inflammatoriese interaksies van CGS 21680 met neutrofiele geneutraliseer.

Ten slotte is my resutate verenigbaar met 'n rol vir die $A_{2A}R$ in die regulering van die pro-inflammatoriese aktiwiteite van menslike neutrofiele deur bevordering van sikliese AMF-afhanklike sekwestrasie van sitosoliese Ca^{2+}.

Sleutelwoorde:

Adenosien; A_{2A} reseptore; kalsium; kalsium invloei; kalsium uitvloei; CGS 21680; CPA; dexamethasone; elastase; IB-MECA; neutrofiele; superoksied; sikliese AMP; ZM 241385.

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A	adenosine
AA	arachidonic acid
A1	member of family of apoptotic regulators
A_1R	adenosine-1 receptor
$A_{2A}R$	adenosine-2A receptor
$A_{2B}R$	adenosine-2B receptor
A_3R	adenosine –3 receptor
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANCA	anti-neutrophil cytoplasmic antibody
ANOVA	analysis of variance
AP-1	activator protein-1
Apo1	apoptosis receptor-1
AR	adenosine receptor
ARDS	adult respiratory distress syndrome
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
AV	atrio-ventricular
BH1,2	anti-apoptotic regions in the Mcl-1 gene
BH3	pro-apoptotic region in the Mcl-1 gene
BPI	Bactericidal permeability increasing protein
C	Complement
cAMP	adenosine 3',5'-cyclic monophosphate
($[Ca^{2+}]i$)	cytosolic free Ca^{2+}
CB	cytochalasin B
CBP	CREB binding protein
CD30L	CD30 ligand
Ced-9	Caenorhabditis elegans cell death gene
cGMP	cyclic guanosine monophosphate

CGS 21680	2(4-[(2-carboxyethyl)phenyl]ethylamino)-5′-N-ethylcarboxamidoadenosine
CINC	cytokine-induced chemoattractants
COX2	cyclooxygenase 2
CPA	N^6-cyclopentyladenosine
$cPLA_2$	cytosolic phospholipase A_2
CREB	cyclic AMP response element binding factor
DMA	N,N-dimethylacetamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EP receptor	Prostaglandin E receptor
FAD	flavine-adenine-dinucleotide
Fas	CD 95 or Apo1
FasL	Fas ligand
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
FURA-2/AM	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl] –2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′,-tetraacetic acid-acetoxy methylester oil
GC	glucocorticoid
G-CSF	granulocyte-colony stimulating factor
GDP	guanosine diphosphate
GM-CSF	Granulocyte macrophage-colony stimulating factor
$gp91^{phox}$	glycoprotein phagocyte oxidase, 91 kDa molecular weight.
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRO-α	growth-related gene product-alpha
GRO-β	growth-related gene product-beta
GTP	guanosine triphosphate
HAT	histone acyltransferase
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HGF	hepatocyte growth factor
HNP1,2,3	human neutrophil peptides 1,2 and 3

H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
Hsp	heat shock protein
IB-MECA	N^6-3-iodobenzyl-5′-N-methylcarbamoyladenosine
ICAM	intercellular adhesion molecule
IFN-γ	interferon-gamma
IFN-β	interferon-beta
Iκ-B-α	inhibitor protein-kappa B-alpha
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
JAM	junctional adhesion molecule
kDa	kiloDalton
LECL	lucigenin-enhanced chemiluminescence
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukaemia gene product, member of family of apoptotic regulators
Mcl-1S/Δ TM	isoform of Mcl-1 with pro-apoptotic characteristics
MCP-1,2,3	monocyte chemotactic protein-1,2,3
M-GSF	macrophage-colony stimulating factor
MIP-1α	macrophage infiltrating protein-1alpha
MIP-1β	macrophage infiltrating protein-1beta
$MnCl_2$	Manganese chloride
MPO	myeloperoxidase
mRNA	messenger RNA

NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NECA	5′-N-ethylcarboxamidoadenosine
NF-κB	nuclear factor-kappa B
nGRE	negative glucocorticoid response element
NO	nitric oxide
NSF	N-ethylmaleimide-sensitive fusion
O_2^-	superoxide anion
•OH	hydroxyl radical
OSM	oncostatin
p300	300 kDa polypeptide
PAF	platelet-activating factor
PAPA-APEC	2-[4-[2-[2-[phenylmethylcarbonylamino]ethylaminocarbonyl] ethyl]phenyl]ethylamino-5′-N-ethyl-carboxamidoadenosine
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PECAM	platelet endothelial cell adhesion molecule
PGE_2	prostaglandin E_2
PKA	protein kinase A
PKC	protein kinase C
PL A_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol-12-myristate 13- acetate
PMN	polymorphonuclear leucocyte
$P22^{phox}$	protein/polypeptide phagocyte oxidase, 22 kDa molecular weight
$P40^{phox}$	protein/polypeptide phagocyte oxidase, 40 kDa molecular weight
$P47^{phox}$	protein/polypeptide phagocyte oxidase, 47 kDa molecular weight
$P67^{phox}$	protein/polypeptide phagocyte oxidase, 67 kDa molecular weight

Rac2	ribosome-associated complex, a member of the Ras superfamily
Raf	a serine kinase linking Ras activation with the nucleus
RANTES	"regulated on activation, normal T-cell expressed and secreted"chemokine
Rap1A	a member of the Ras superfamily
Ras	rat sarcoma gene product, a superfamily of GTP-binding proteins
Rho	a family of G-proteins
RNA	ribonucleic acid
SCF	stem cell factor
SEM	standard error of the mean
SNARES	soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor
SNAP-23	synaptosomal-associated protein of 23 kDa.
STATs	signal transducers and activators of transcription
STZ	serum-treated zymosan
TGF-α,β	tumour growth factor-alpha, beta
TNF-α	Tumour necrosis factor-alpha
TPA	12-0-tetradecanoylphorbol-13-acetate
t-SNARES	target plasma membrane protein-SNARES
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-2,5,6,9	very late activation protein-2,5,6,9
v-SNARES	vesicle proteins-SNARES
ZM 241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5yl amino]ethyl)phenol