Nitric oxide and central autonomic control of blood pressure: A neuroanatomical study of nitric oxide and cGMP expression in the brain and spinal cord

Kellysan Powers-Martin

This thesis was submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Biomedical Science), Murdoch University.

July 2008

Declaration

I declare that this thesis is my own account of my research and contains work that has not been previously submitted for a degree at any other tertiary educational institution.

Kellysan Powers-Martin

Abstract

Essential hypertension is defined as a chronic elevation of blood pressure of unknown cause. Though a definitive trigger for this change in blood pressure has not been established, there is a strong association with an upregulation of sympathetic output from the central nervous system. There are a number of central autonomic nuclei involved in the maintenance of blood pressure, including the brainstem regions of the nucleus tractus solitarii (NTS), caudal ventrolateral medulla (CVLM), rostral ventrolateral medulla (RVLM), the sympathetic preganglionic neurons (SPNs) within the intermediolateral cell column (IML) of the spinal cord, as well as forebrain regions such as the paraventricular nucleus (PVN) of the hypothalamus. Within these centers, a vast number of neurotransmitters have been identified that contribute to the control of blood pressure, including glutamate, angiotensin II, serotonin, neurotensin, neuropeptide Y, opioids and catecholamines. Recognition of the role of nitric oxide (NO) and its multiple influences over the neural control of blood pressure is gaining increasing significance.

Nitric oxide is a unique modulatory molecule that acts as a non-conventional neurotransmitter. As NO is a gas with a short half-life of 4 – 6 seconds, its' synthesising enzyme, nitric oxide synthase (NOS) is often used as a marker of location of production. Once activated, the best-known "receptor" for NO is soluble guanylate cyclase (sGC), which drives the production of cyclic guanosine monophosphate (cGMP). Identifying the presence of cGMP can therefore be used to determine sites receptive to NO. Previous studies examining the role of NO in the central autonomic control of blood pressure have focused predominantly upon application of either excitatory or inhibitory drugs into the key central autonomic regions and assessing pressor or depressor effects. This thesis aims instead to study

the neuroanatomical relationship and functional significance of NO and cGMP expression in the brain and spinal cord of a hypertensive and normotensive rat model.

In the first experimental chapter (Chapter 3), a comparative neuroanatomical analysis of neuronal NOS expression and its relationship with cGMP in the SPN of mature Spontaneously Hypertensive Rats (SHR) and their controls, Wistar Kyoto (WKY) was undertaken. Fluorescence immunohistochemistry confirmed the expression of nNOS in the majority of SPN located within the IML region of both strains. However, a strain specific anatomical arrangement of SPN cell clusters was evident and while there was no significant difference between the total number of SPN in each strain, there were significantly fewer nNOS positive SPN in the SHR animals. All nNOS positive SPN were found to express cGMP, and a novel subpopulation of nNOS negative, cGMP-positive SPN was identified. These cells were located in the medial edge of the IML SPN cell group. These results suggest that cGMP is a key signalling molecule in SPN, and that a reduced number of nNOS positive SPN in the SHR may be associated with the increase in sympathetic tone seen in essential hypertension.

The second experimental chapter (Chapter 4) aimed to determine if reduced numbers of nNOS containing SPN translated into reduced detectable cGMP. The functional significance of cGMP signalling in the two strains was then examined. Based on previous work by our group, it was predicted that reduced nNOS in the SHR would translate into reduced cGMP and that intrathecal administration of exogenous cGMP in the spinal cord would drive a differential pressor response in the two animal strains. Immunohistochemical techniques confirmed that within each SPN, the relative level of cGMP expression was significantly reduced in the SHR when compared to the WKY. Intrathecal application of 8-bromo-cGMP, a drug

analogous to cGMP, increased blood pressure in both strains and had a differential and dose dependent effect, causing only a small increase in blood pressure in anaesthetised WKY animals, while driving a significant pressor response in the SHR. This finding raised the novel hypothesis that in the SHR, reduced nNOS expression is not a driver of hypertension, but is instead a protective mechanism limiting the potent pressor effects of cGMP within SPN.

The third experimental chapter (Chapter 5) examines the expression of neuronal and inducible isoforms of NOS (nNOS, iNOS) within the RVLM of SHR and WKY rats. Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyse the level of mRNA expression and immunohistochemistry was then used to further analyse protein levels of nNOS. Total RNA was extracted and reverse transcribed from the RVLM of mature male WKY and SHR. Quantitative real-time PCR indicated that relative to WKY, mRNA levels for nNOS was significantly higher in RVLM of the SHR. This was confirmed immunohistochemically. When compared to iNOS, nNOS was expressed at significantly higher levels overall, however there was no difference in iNOS mRNA expression between the two strains. This demonstration of differential expression levels of nNOS and iNOS in the RVLM raises the possibilities that (i) NO production is up-regulated in the RVLM in SHR in response to increased sympathetic activity in order to re-establish homeostatic balance or alternatively that (ii) an alteration in the balance between nNOS and iNOS activity may underlie the genesis of augmented sympathetic vasomotor tone during hypertension.

The fourth experimental chapter (Chapter 6) extends the observations in Chapter 5 through examination of the expression of cGMP and sGC within the RVLM. There is strong functional evidence to suggest that NO signalling in the

RVLM relies on cGMP as an intracellular signalling molecule and that this pathway is impaired in hypertension. Immunohistochemistry was used to assess cGMP expression as a marker of active NO signalling in the C1 region of the RVLM, again comparing SHR and WKY animals. Fluorescence immunohistochemistry on sections of the RVLM, double labelled for cGMP and either nNOS or phenylethylamine methyl-transferase (PNMT) failed to reveal cGMP positive neurons in the RVLM from aged animals of either strain, despite consistent detection of cGMP immunoreactivity neurons in the nucleus ambiguus from the same or adjacent sections. This was demonstrated both in the presence and absence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) and in young vs. aged animals. *In-vitro* incubation of RVLM slices in the NO donor DETA-NO or NMDA did not reveal any additional cGMP neuronal staining within the RVLM. In all studies, cGMP was prominent within the vasculature. Soluble guanylate cyclase immunoreactivity was found throughout the RVLM, although it did not co-localise with the PNMT or nNOS neuronal populations. Overall, results suggest that within the RVLM, cGMP is not detectable in the resting state and cannot be elicited by phosphodiesterase inhibition, NMDA receptor stimulation or NO donor application. A short time course of cGMP signalling or degradation not inhibited by the phosphodiesterase inhibitor utilised (IBMX) in the RVLM cannot be excluded.

The final experimental chapter (Chapter 7) examines cGMP expression in magnocellular and preautonomic parvocellular neurons of the PVN. Retrograde tracing techniques and immunohistochemistry were used to visualise cGMP immunoreactivity within functionally, neurochemically and topographically defined PVN neuronal populations in Wistar rats. Basal cGMP immunoreactivity was readily observed in the PVN, both in neuronal and vascular profiles. Cyclic GMP

immunoreactivity was significantly higher in magnocellular compared to preautonomic neuronal populations. In preautonomic neurons, the level of cGMP expression was independent on their subnuclei location, innervated target or neurochemical phenotype. The data presented in this chapter indicates a highly heterogeneous distribution of basal cGMP levels within the PVN, and supports work by others indicating that constitutive NO inhibitory actions on preautonomic PVN neurons are likely mediated indirectly through activation of interneurons.

Summary

Together, these studies comprise a detailed analysis of the neuroanatomical expression of NO and its signalling molecule cGMP in key central autonomic regions involved in the regulation of blood pressure. Under resting or basal conditions, the studies demonstrate notable differences in the expression of NO synthesising enzymes between normotensive and hypertensive animals, and correlating changes in the downstream signalling molecule cGMP. In the spinal cord, novel functional differences in cGMP activity were also demonstrated. In the RVLM, although differences in nNOS were demonstrated, cGMP expression could not be readily detected in either the WKY or SHR, while in contrast within the PVN, cGMP was detected in both magnocellular and parvocellular neuronal populations.

Conclusion

This thesis gives insight into the physiological role of NO and cGMP as mediators of central blood pressure control. The results presented indicate that the NO-cGMP dependent signalling pathway may not be the dominant driver responsible for maintaining high blood pressure in the SHR model of essential hypertension, and that there is no globally consistent pattern of expression, and indeed the role of NO as a mediator of pressor and depressor function may vary between the autonomic

regions examined. Further, it is possible that this pathway is only recruited during activation of reflex homeostatic pathways or during times of marked physiological stress, and that the differences we see in basal expression between the normotensive and SHR animals are instead a result of compensatory mechanisms.

Acknowledgements

What to say without sounding cliché? There are of course the obvious thank-yous – Jackie for driving me nuts, Doug for always sounding terrified of me on the phone, and Leonard for….ummm, what do you look like again? Ok, just joking. Jackie you are an amazingly intelligent person who I truly admire. Doug, though you don't say much, what you do say is gold. Leonard, you always strive to make me think

However, a thesis is an embodiment of work for which many more people need to be given credit for.

To my fellow colleagues – thank you all for your various inputs. I can't believe there are so many of you, Paula, Anna, Yelena, Courtney, Raihana, Su Ann, Little Jo, Gael, Niloufer and John – thanks for all the good times. Rhonda – thankyou thank you, thank you for your patience and talent to teach; Steve, thanks for all the IT assistance. Varun and Vasyl, you were a pleasure to work with. To the current inhabitants of our office, Ben, Kathryn, Penny and Tammy, thank-you so much for this year – I would have never made it without you. Words cannot say how much you guys mean to me. I will miss the good times, our lovely "pets", and the constant support. A big thank-you has to go to Trish, as well – for all the times she's propped me up this year. Lastly, Susannah – who has made sure I finish these last 18 months, no matter how much I wanted to give up. SusieQ, you're one of my closest friends, my best friend through my PhD.

Another set of thanks has to go to the wonderful people I have met interstate and internationally, Anne Goodchild, Paul Pilowsky, Deborah Spingell, Javier Stern and Vinicia Biancardi. Anne, you are an amazing scientist, with such patience I'll never forget and Paul, thank you for your invaluable insight. Deb, thankyou for all your hard work. The three of you should all take credit in helping me become the scientist I am today. To Javier and Vinicia, thank you for helping me feel so welcome in the USA, and for your input and advice on everything.

To my awesome friends, thank-you for keeping me sane and grounded, without your patience, coffees, dinners and so forth, I would surely have lost the plot. A big thank-you to my closest friends, in no particular order Grace, Cindy, Lee Lee,

David, Jacqui, Catherine, Andrea, Emily, Emma and Lorraine I owe you all afternoon tea and bickies.

Lastly, to my wonderful family. My mum, for giving me my fighting spirit, and my dad for giving me that balancing calm. I love you both so much, and just want you to say thank-you for everything. My little family of animals, thank-you for those cuddles, particular Lily and Saffy. Lastly, a huge thank-you goes to Rob, words cannot express how much you mean to me. You have seen me at my best and my worst, and you were always there, still loving me and picking up the pieces. I will never be able to thank-you enough. Oh and I need help formatting this….so don't go anywhere yet. And can I have my thumb drive back please? Thanks hunny.

"Science has made us gods

even before we are worthy of being men"

Jean Rostand – French biologist (1894 – 1977)

Publications arising from this thesis

Edwards MA, Loxley RA, **Powers-Martin K**, Lipski J, McKitrick DJ, Arnolda LF, Phillips JK.

Unique levels of expression of N-methyl-D-aspartate receptor subunits and neuronal nitric oxide synthase in the rostral ventrolateral medulla of the spontaneously hypertensive rat.

Brain Research: Molecular Brain Research 2004; 129(1-2): 33-43.

Powers-Martin K, McKitrick DJ, Arnolda LF, Phillips JK.

Distinct subpopulations of cyclic guanosine monophosphate (cGMP) and neuronal nitric oxide synthase (nNOS) containing sympathetic preganglionic neurons in spontaneously hypertensive and Wistar-Kyoto rats.

The Journal of Comparative Neurology 2006; 497(4): 566-74.

Powers-Martin K, Barron AM, Auckland C, McCooke J, McKitrick DJ, Arnolda LF, Phillips JK

Immunohistochemical assessment of cyclic guanosine monophosphate (cGMP) and soluble guanylate cyclase (sGC) within the rostral ventrolateral medulla

Journal of Biomedical Science 2008; 15(6): 801-12.

Powers-Martin K, Phillips JK, Biancardi VC, Stern JE.

Heterogeneous Distribution of Basal Cyclic Guanosine Monophosphate (cGMP) within Distinct Neuronal Populations in the Hypothalamic Paraventricular Nucleus

American Journal of Physiology: Regulatory, Integrative and Comparative Physiology 2008; 295(4): R1341-50.

Other publications arising from period of candidature

Springell DA, **Powers-Martin K**, Phillips JK, Pilowsky PM, Goodchild AK.

Phosphorylated extracellular signal-regulated kinase 1/2 immunoreactivity identifies a novel subpopulation of sympathetic preganglionic neurons.

Neuroscience. 2005; 133(2): 583-90.

Table of Contents

List of Figures

List of Tables

Abbreviations

2K1C	2 kidney 1 clip
8-Br-cGMP	8-Bromo-cGMP
A1	adrenergic groups
aCSF	artificial cerebrospinal fluid
ADH	antidiuretic hormone
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Ang II	angiotensin II
ANP	atrial
ANS	autonomic nervous system
anti α-SMA	anti a smooth muscle actin
AVP	arginine vasopressin
BH_4	(6R)-6,5,7,8-tetrahydrobiopterin
BNP	brain natriuretic peptide
BP	blood pressure
CAA	central autonomic area
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
ChAT	choline acetyl transferase
cNOS	constitutive NOS
CNP	C-type natriuretic peptide
CNS	central nervous system
CO_2	carbon dioxide
CRH	corticotropic releasing hormone
CSN	commissural subnucleus
Ct	cycle threshold
CVLM	caudal ventrolateral medulla
CY2	cyanine
CY3	indocarbocyanine
CY5	indodicarbocyanine
DAB	diaminobenzidase
DAF-2	4,5-diaminofluorescein-2-diacetate
DBH	dopamine b oxidase
DC	dorsal cap
dNTPs	deoxynucleoside-triphosphate
eNOS	endothelial NO synthase
ERK	extracellular signal-related kinase
FG®	Fluorogold®
FITC	fluorescein isothiocyanate
GABA	g-aminobutyric acid
GCs	guanylyl cyclases
HIF-1α	hypoxic inducible factor 1 alpha
HR	heart rate
IBMX	inhibitor isobutylmethylxanthine

IM	intramuscular
IML	intermediolateral lateral cell column
io	inferior olive
IP	intraperitoneal
IR	immunoreactivity
L-arg	L-arginine
LM	lateral magnocellular
MAP	mean arterial pressure
MM	medial magnocellular
mRNA	messenger RNA
NA	nucleus ambiguus
NaN_3	sodium azide
NAPDH	nicotinamide-adenine-dinucleotide phosphate
NMDA	N-methyl-D-aspartate
nNOS	Neuronal NOS
NO	nitric oxide
NOS	NO synthase
NR1	N-methyl-D-aspartate receptor 1
NR2	N-methyl-D-aspartate receptor 2
NRS	normal rabbit serum
NSE	neuronal specific enolase
NTS	nucleus tractus solitarii
ODQ	1H-[1,2,4] oxadiazole [4,3-a] quinoxaline-1-one
OT	oxytocin
OVLT	organum vascularis lamina terminalis
PaPo	posterior parvocellular
PB	phosphate buffer
PDE	phosphodiesterases
p-ERK1/2	phosphorylated extracellular signal regulated kinase 1/2
PKG	protein kinase G
PNMT	phenylethanolamine-n-methyltransferase
PPE	preproenkephalin
PSD95	post synaptic density protein
PVN	paraventricular nucleus of the hypothalamus
RSNA	renal sympathetic nerve activity
RT-PCR	polymerase chain reaction
RVLM	rostral ventrolateral medulla
sd	standard deviation
SEM	standard error of the mean
SFO	subfornical organ
sGC	soluble guanylate cyclase
SHR	Spontaneously Hypertensive Rat
SND	sympathetic nervous discharge
SON	supraoptic nucleus
SPN	sodium nitroprusside
SPN	sympathetic preganglionic neurons

TH	tyrosine hydroxylase
TPBS	Tris phosphate buffered saline
VM	ventromedial parvocellular
WKY	Wistar Kyoto