DEFINING THE ROLE(S) OF NOX2-NADPH OXIDASE IN THE CEREBRAL CIRCULATION

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Bachelor of Biomedical Science (Honours)

A thesis submitted to the Faculty of Medicine, Nursing and Health Sciences

in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

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ERRATA

P 10 legend for Figure 2 line 2: Replace “if” with “of”

P 32 2nd paragraph, line 4: “lucigenin-enhanced” for “lucigenin-enhance”

P 67, line 4: Delete “several” so it reads “...from a number of animal species”

P 71, section 1, line 1: Delete “consistently”

P 89, 2nd last line: Delete “consistently”

P 111, first line: “suggest” for “suggests”

P 111, last line: “much less is known” for “much less in known”

P 119, 3rd last line: Delete “consistently”

ADDENDUM

P 2 from 6th last line: edit so it reads “During increased neural activity, metabolites (e.g. adenosine) are released by neurons and activate receptors on the endothelium (Iadecola, 2004). This results in vasoactive agents being released from endothelial cells and dilatation of local and upstream cerebral arteries, thus increasing blood flow to that region of the brain.”

P 9 line 9: Insert “it” after “For example,“ so it reads “For example, it...”

P 11 2nd last lines: Insert “(unstimulated)” after “resting cells”

P 11 last line: Insert “(unstimulated)” after “Under resting conditions”

P 14 legend for Figure 3 line 9: “PKC phosphorylates p47phox displacing the autoinhibitory region (AIR) and thus, revealing the p22phox binding site.” For “PKC phosphorylates p47phox revealing the p22phox binding site”

P 18 2nd paragraph, line 2: Insert “page 4” after “see *Reactive Oxygen Species and Cerebrovascular Tone”*

P 22 middle paragraph line 7/8: replace “...neurons were functionally inactive but still alive” with “...neurons retained structural integrity but were functionally inactive”

P 24 middle paragraph end of first sentence: Insert reference “(Denes *et al.,* 2009)”.

P 27, at the start of the first paragraph: Insert “The overall hypothesis of this thesis is that Nox2-NADPH oxidase is the cause of cerebral vascular dysfunction that is associated with

high risk cardiovascular states, such as hypertension and hypercholesterolaemia, and following ischaemic stroke."

P 34, 2nd section, line 6: Insert "Vessels were checked for leaks by switching the pressure servo unit to 'manual' mode, which monitored, but did not regulate pressure." after "...without further intraluminal perfusion."

P 34, 2nd section, line 8: Insert "Vessels that did not achieve a maximum constriction of > 50 % to KPSS were excluded." after "...(equimolar replacement of NaCl with KCl)."

P 34 last paragraph, 1st line: Insert "(typically 2 MCA and 1 basilar artery was used per n)" after "Cerebral arteries" so that it reads "Cerebral arteries (typically 2 MCA and 1 basilar artery was used per n) were excised..."

P 38: At the end of "Data Analysis" section, insert "All statistical analyses were performed using Graphpad Prism version 5.03 software (Graphpad Software Inc.)"

P 42 2nd paragraph, line 1: Replace "(pooled basilar and MCA)" with "(1 MCA and half of a basilar artery)".

P 46 2nd paragraph, at the end of first sentence: Insert "Intraluminal diameters were measured under steady-state conditions (at 60 mmHg), prior to commencing the concentration response curve under."

P 52, last line: Delete "with a Bonferroni multiple comparison post-hoc test"

P 54, line 8/9: Delete "with a Bonferroni multiple comparison post-hoc test"

P 69 2nd paragraph, line 2: Replace "(pooled basilar and MCA)" with "(1 MCA and half of a basilar artery)".

P 73, last 2 lines: Delete "with a Bonferroni multiple comparison post-hoc test"

P 77, last 2 lines: Delete "with a Bonferroni multiple comparison post-hoc test"

P 80, 12th last line: Insert "Further studies are needed to determine if H_2O_2 directly activates Rho-kinase to elicit constriction of MCA." after "...to elicit constriction of MCA."

P 80, after above comment: Insert "It may be possible that phospholipase C-derived DAG and/or PKC activates both Nox2-NADPH oxidase and the RhoA/Rho-kinase pathway independently (via Gαq/11 and RhoA) and thus, despite both being able to elicit constriction, these signalling pathways may not be connected."

P 85 first paragraph, line 7: Replace “background” with “strain” so that is reads “...were of the C57Bl6/J strain”

P 86 last paragraph, first line: Replace “(pooled basilar and MCA)” with “(1 MCA and half of a basilar artery)”.

P 105, line 13: Insert “[released in response to various stimuli, such as shear stress *in vivo* (Busse & Fleming, 2003)]” after “...steady-state levels of endothelial-derived NO·...”

P 106, line 8: Insert “In the presence of tempol, the magnitude of L-NAME induced constriction was reduced (Figure 6A vs. Figure 7A). This is likely due to the fact that in these experiments, MCA from wild type and $ApoE^{-/-}$ mice were studied in parallel. As such, there was a delay in studying these vessels due to the increased setup time and it is conceivable that this contributes to the reduced magnitude of L-NAME-induced constriction. Nevertheless, these findings clearly demonstrate that the reduced NO·-function is likely due to acute scavenging of NO· by O_2^-.” Before “It is also conceivable that...”

P 106, line 17: Replace “unlikely to” with “may not”.

P 118, last line: “Oedema volume was not different between wild type and $Nox2^{-/-}$ mice” for “There was a trend for oedema volume to be smaller in $Nox2^{-/-}$ compared with wild type mice, however, this failed to reach statistical significance”.

P 119, line 3: Insert “(ipsilateral)” after “Ischaemic”

P 119, line 4: Insert “(contralateral)” after “non-Ischaemic”

P 126, last 2 lines: Delete “with a Bonferroni multiple comparison post-hoc test”

P 132, Line 4: Replace “as NOS inhibition augments vasocontraction to phenylephrine” with “as NOS inhibition or disruption of the endothelium augments vasocontraction to α-adrenoceptor agonists”. Insert new references: Carrier *et al.,* 1993; Dora *et al.,* 1997; Egleme *et al.,* 1984; Kaneko & Sunano, 1993.

P 133, Line 3: Insert ”It is also conceivable that O_2^- and/or a downstream ROS, such as hydrogen peroxide, might impair NO· signalling by reducing soluble guanylate cyclase activity following ischaemia/reperfusion (Gerassimou *et al.*, 2007; Priviero *et al.*, 2009).”

P 140, 2nd paragraph, line 4: Insert “As mentioned in previous sections of this thesis, previous work in cerebral endothelial cells has demonstrated that H_2O_2 can activate the

RhoA/Rho-kinase pathway, eliciting constriction (Kahles *et al.,* 2007)" after "...angiotensin II via the RhoA/Rho-kinase pathway."

P 153: New reference "Busse, R, Fleming, I (2003) Regulation of endothelium-derived vasoactive autacoid production by hemodynamic forces. *Trends in Pharmacological Sciences* **24**(1)**:** 24-29."

P 153: New reference "Carrier, GO, White, RE (1985) Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *Journal of Pharmacology and Experimental Therapeutics* **232**(3)**:** 682-687."

P 156: New reference "Denes, A, Thornton, P, Rothwell, NJ, Allan, SM (2009) Inflammation and brain injury: Acute cerebral ischaemia, peripheral and central inflammation. *Brain, Behavior, and Immunity* **24**(5)**:** 708-723."

P 157: New reference "Dora, KA, Doyle, MP, Duling, BR (1997) Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. *Proceedings of the National Academy of Sciences* **94**(12)**:** 6529-6534."

P 157: New reference "Egleme, C, Godfraind, T, Miller, RC (1984) Enhanced responsiveness of rat isolated aorta to clonidine after removal of the endothelial cells. *British Journal of Pharmacology* **81**(1)**:** 16-18."

P 161: New reference "Iadecola, C (2004) Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nature Reviews: Neuroscience* **5**(5)**:** 347-360."

P 164: New reference "Kaneko, K, Sunano, S (1993) Involvement of alpha-adrenoceptors in the endothelium-dependent depression of noradrenaline-induced contraction in rat aorta. *European Journal of Pharmacology* **240**(2-3)**:** 195-200."

Table of Contents

SUMMARY

Evidence suggests that NADPH oxidase-derived reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are important signalling molecules within cerebral arteries. However, if there is an imbalance between ROS production and metabolism oxidative stress can ensue. One of the major consequences of oxidative stress is a decrease in the bioactivity of the vasoprotective molecule nitric oxide (NO·). This thesis examined the role of cerebrovascular Nox2-containing NADPH oxidase in (1) gender-dependent differences in responses to angiotensin II in mice; and (2) excessive ROS production and diminished NO· function in mouse models of hypercholesterolaemia and ischaemic stroke.

The major experimental approaches used in this thesis were 1) chemiluminescence and fluorescence techniques for the measurement of ROS production; 2) perfusion myography for the assessment of NO· function *in vitro*; and 3) western blotting and immunofluorescence for the localization and measurement of various proteins of interest.

In Chapter 3, we demonstrate that cerebrovascular ROS production and constrictor responses to angiotensin II are considerably smaller in female versus male wild type (WT) mice. In male Nox2-deficient ($Nox2^{-/-}$) mice, responses to angiotensin II were attenuated, whereas Nox2 deletion had no effect on responses in female mice. These Nox2-related gender-dependent differences were not associated with differences in Nox2 protein expression or cellular localization. Taken together, these data demonstrate for the first time that these gender-dependent differences in responses to angiotensin II are likely to be due to lower Nox2 activity in females. Another novel finding of Chapter 3 was that Nox2-derived ROS is critical mediator of angiotensin II-induced constrictions in the cerebral circulation of male mice. Therefore, in Chapter 4 we sought to determine the identity of the ROS responsible. Using pharmacological scavengers of O_2^- and H_2O_2 we found evidence that H_2O_2 (or a downstream ROS) mediates constrictions to angiotensin II in the mouse cerebral circulation.

In Chapter 5, we found that despite the absence of atherosclerotic lesions, cerebrovascular O_2^- production was augmented and NO· function was impaired in hypercholesterolaemic apolipoprotein E-deficient ($ApoE^{-/-}$) mice versus WT mice. However, in $Nox2^{-/-}/ApoE^{-/-}$ mice, cerebrovascular O_2^- production was not elevated and NO· function was comparable to that in WT mice. Protein expression of Nox2 was not elevated in $ApoE^{-/-}$ mice, however, expression of p47phox (Nox organizer) was 2-fold higher in cerebral arteries from $ApoE^{-/-}$ mice. Therefore, this study demonstrates that excessive O_2^- production and impaired by NO· function occur in cerebral arteries of hypercholesterolaemic mice, as a consequence of increased activity of Nox2-NADPH oxidase.

In Chapter 6, we found that at 24 h after middle cerebral artery occlusion in WT mice, O_2^- production is augmented and NO· function is impaired in ischaemic versus non-ischaemic middle cerebral arteries (MCA). By contrast, in $Nox2^{-/-}$ mice, cerebrovascular O_2^- production and NO· function was comparable between ischaemic and non-ischaemic MCA. Thus, Nox2-NADPH oxidase plays a central role in cerebrovascular NO· dysfunction following ischaemia and reperfusion.

In summary, the findings of this thesis indicate that Nox2-derived ROS plays a key role in mediating cerebrovascular responses to angiotensin II and in mediating impaired NO· function during hypercholesterolaemia and following stroke. While future work is needed to determine whether similar changes occur in humans and in other vascular diseases, these findings position Nox2-NADPH oxidase as a strong candidate for the excessive ROS production that is thought to lead to oxidative stress associated with a number of vascular diseases. As such, we propose that Nox2-NADPH oxidase may represent a therapeutic target to alleviate the burden of oxidative stress-induced vascular dysfunction.

DECLARATION

In accordance with the Monash University Doctorate Regulation 17 - Doctor of Philosophy and Master of Philosophy (MPhil) regulations, the following declarations are made:

I hereby declare that this thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge it contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I wish to acknowledge the assistance of Dr Brad Broughton who performed the Nox2 immunofluorescence experiments (Chapter 3, Figure 8) and Mr Henry Diep who performed *en face* oil red O staining of aortae (Chapter 5, Figure 3C-D).

The core theme of the thesis is "Defining the role(s) of Nox2-NADPH oxidase in the cerebral circulation". The ideas, development and writing up of all chapters in this thesis were the principal responsibility of myself under the supervision of Dr Alyson Miller and Associate Professor Christopher Sobey.

Signed:_____ Date:_____

Travice Michael De Silva

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors, Dr Alyson Miller and Associate Professor Christopher Sobey, without all of your help, guidance, enthusiasm and patience, I would never have made it this far. Alyson, thank you for everything that you have done over the last 5 years. When I started, you said that this was a first for the both of us and we will both be learning together, well we made it through pretty well. Thanks for being so patient (I still think there might have been a push down) and always willing to see me and work through any problem I was having. Lastly, even though sometimes you may not think so, I do listen to everything that you say! Chris, you have always managed to put a positive spin on any bad news and have always had a good suggestion whenever things were not going as planned. I one day hope that I might be able to emulate the both of you and have a truly successful and meaningful scientific career.

Thank you to Dr Brad Broughton for his help and advice when strokes didn't quite go to plan as well as for performing the Nox2 immunofluorescence in Chapter 3 and being the go-to man for all things histology. Also, thank you to Henry Diep for performing and analysing the *en face* oil red O staining of aortae performed in Chapter 5. A special thank you to (Dr to be) Vanessa Brait for teaching me how to perform the stroke surgery and for all of your encouragement over the last few months of writing. I am sure everything will work out and I look forward to visiting you in Barcelona!

Thank you to all of the members of the VBIG lab as well as the entire Department of Pharmacology (both past and present). There are too many people to thank individually but thanks for: coffee runs, morning/lunchtime/afternoon chats in the corridors, helpful advice (both regarding science and life in general), supercoach chat, for making me laugh and so much more. Your help over the last 5 years has been invaluable, and your friendship has made coming to work enjoyable. A special mention to Craig Harrison for his odd bits of daily news, humour on late nights and weekends at work as well as the for the daily coffee/energy drink run during the last couple of months.

To my friends outside of the lab, Craig, Andrew, Emmett, Louie and Ann. Thanks for helping to get my mind off of work when I needed it. For the talks and the laughs and reminding me to still try and enjoy life. The catch ups for coffee, lunch or to let out some frustration on a golf ball or two, it has helped me keep my sanity.

To my family, both De Silva and Lo, thank you for all of your support and understanding. I may not have been the most helpful son or brother over the last few months; I will make up for it. A special mention to my parents, your support has helped me to get through. You both have taught me to never give up and always try my hardest even when things are not going well. I will always remember that nothing is impossible.

Finally, but by no means least, a very special thank you to my absolutely amazing fiancée, Cecilia. Your love and motivation has kept me going, especially during these last few weeks. Thanks for not asking too much of me during the last few months of writing and for keeping me company on the weekends and during some of the late nights at work.

PUBLICATIONS ARISING FROM THIS THESIS

Manuscripts

MILLER A.A., **DE SILVA T.M.**, JACKMAN K.A. & SOBEY C.G. (2007) Influence of gender and sex hormones on vascular oxidative stress, *Clin Exp Pharmacol Physiol*. 34:1037-1043.

MILLER A.A., DRUMMOND G.R., **DE SILVA T.M.**, MAST A.E., HICKEY H., WILLIAMS J. & SOBEY C.G. (2008) NADPH oxidase activity is higher in cerebral arteries: role of Nox2, *Am J Physiol Heart Circ Physiol*. 296 (1):H220-5.

DE SILVA T.M., BROUGHTON B.R.S., DRUMMOND G.R., SOBEY C.G. & MILLER A.A. (2009) Gender Influences cerebral vascular responses to angiotensin II via Nox2-derived reactive oxygen species, *Stroke*. 40 (4):1091-1097.

JACKMAN K.A., MILLER A.A., **DE SILVA T.M.**, CRACK P., DRUMMOND G.R. & SOBEY C.G. (2009) Reduction of cerebral infarct volume by apocynin requires pretreatment and is absent in Nox2-deficient mice, *Br J Pharmacol*. 156 (4):680-688.

MILLER A.A*., **DE SILVA T.M.*,** DRUMMOND G.R., DIEP H., JUDKINS C.J. & SOBEY C.G. (2010) Augmented superoxide production by Nox2-containing NADPH-oxidase causes cerebral artery dysfunction during hypercholesterolemia, *Stroke*. 41 (4):784-789. **Both authors contributed equally to this study.*

Conference Abstracts

1. **DE SILVA T.M.**, SOBEY C.G. & MILLER A.A. (2007). Gender influences angiotensin II signalling in the cerebral circulation. *Frontiers in Vascular Medicine*, Abstract #108.

2. **DE SILVA T.M.**, SOBEY C.G. & MILLER A.A. (2007). Gender influences angiotensin II signalling in the cerebral circulation. *Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists and Southeast Asian Western Pacific Regional Meeting of Pharmacologists,* Abstract #56.

3. **DE SILVA T.M.**, BROUGHTON B.R.S., DRUMMOND G.R., SOBEY C.G. & MILLER A.A. (2008). Gender Influences cerebral vascular responses to angiotensin II via Nox2-derived reactive oxygen species, *Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists,* Abstract #306.

4. **DE SILVA T.M.**, BROUGHTON B.R.S., DRUMMOND G.R., SOBEY C.G. & MILLER A.A. (2008). Gender influences cerebral vascular responses to angiotensin II via Nox2-derived reactive oxygen species and rho-kinase, *High Blood Pressure Research Council of Australia 2008 Annual Scientific Meeting,* Abstract # 86.

5. **DE SILVA T.M.**, BROUGHTON B.R.S., DRUMMOND G.R., SOBEY C.G. & MILLER A.A. (2009). Gender influences cerebral vascular responses to angiotensin II via Nox2-derived reactive oxygen species and rho-kinase, *Experimental Biology 2009*, Abstract # 935.4.

6. MILLER A.A., DRUMMOND G.R., **DE SILVA T.M.**, JUDKINS C.P. & SOBEY C.G. (2009). Excessive superoxide production and endothelial dysfunction in cerebral arteries of atherosclerotic mice occur in the absence of lesions and are due to the activity of Nox2-containing NADPH oxidase, *Experimental Biology 2009*, Abstract # 574.4.

7. **DE SILVA T.M.**, DRUMMOND G.R., BROUGHTON B.R.S., JUDKINS C.P., SOBEY C.G. & MILLER A.A. (2009). Defining the role(s) of Nox2-NADPH oxidase in the cerebral circulation, *63rd High Blood Pressure Research Conference*. Published in: *Hypertension* **54** (4): E32-E32.

8. MILLER A.A., **DE SILVA T.M.**, JUDKINS C.P., DIEP H., DRUMMOND G.R., & SOBEY C.G. (2009). Excessive superoxide production and endothelial dysfunction in cerebral arteries of atherosclerotic mice occur in the absence of lesions and are due to the activity of Nox2-containing NADPH oxidase. *24th International Symposium on Cerebral Blood Flow and Metabolism*. Published in: *J Cereb Blood Flow Metab 2009,* **29**: S73-S73, Suppl. S1.

9. MILLER A.A., **DE SILVA T.M.**, BRAIT V.H., DRUMMOND G.R., & SOBEY C.G. (2010). Excessive superoxide production and endothelial dysfunction in cerebral arteries

following transient cerebral ischaemia are due to enhanced activity of Nox2-containing NADPH oxidase. *Proceeding of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists,* Abstract #p97.

Awards

2008 **Winner of the 'ASCEPT Oral Prize' for best oral presentation by a PhD student,** at the Australian Health & Medical Research Congress, Brisbane, Australia.

2008 **Winner of the 'Best of the Best student poster competition' for best poster presentation by a PhD student at the AHMR congress,** at the Australian Health & Medical Research Congress, Brisbane, Australia.

2008 **Winner of the 'Student Oral Presentation Award' for the best presentation by a PhD student,** at the High Blood Pressure Research Council of Australia Annual Meeting, Melbourne, Australia.

2008 **Winner of the American Council for High Blood Pressure Research award for the best presentation by a PhD student or Post Doctoral researcher,** at the High Blood Pressure Research Council of Australia Annual Meeting, Melbourne, Australia. Prize included a invitation to speak at the *63rd High Blood Pressure Research Conference* in Chicago, USA.

2009 **Second place in the ASPET Cardiovascular Division Runner-Up Poster Competition,** at Experimental Biology 09, New Orleans, USA.

ABBREVIATIONS

ANOVA	analysis of variance
$ApoE^{-/-}$	apolipoprotein E-deficient
AT_1	angiotensin type I receptor
AT_2	angiotensin type II receptor
BH_4	tetrahydrobiopterin
CCA	common carotid artery
cGMP	cyclic guanosine monophosphate
CIV	corrected infarct volume
COX	cyclooxygenase
DAG	diacylglycerol
DETCA	diethyldithiocarbamic acid
DMSO	dimethyl sulfoxide
DPI	diphenyleneiodonium
ECA	external carotid artery
EDRF	endothelium-derived relaxing factor
eNOS	endothelial nitric oxide synthase
EtOH	ethanol
FAD	flavin adenine dinucleotide
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HDL	high density lipoprotein
HRP	horseradish peroxidise
ICA	internal carotid artery
ICAM-1	intercellular adhesion molecule 1
iNOS	inducible nitric oxide synthase
KPSS	high potassium physiological salt solution
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LDL	low density lipoprotein

LHA	left hemisphere area
LIA	left hemisphere infarct area
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein 1
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO·	nitric oxide
NOS	nitric oxide synthase
$Nox2^{-/-}$	Nox2-deficient
O_2^-	superoxide
OH^-	hydroxyl radical
$ONOO^-$	peroxynitrite
OV	oedema volume
OVX	ovariectomy
$p47phox^{-/-}$	p47phox-deficient
PBS	phosphate buffered saline
PDB	phorbol-12, 13 dibutyrate
PI3-K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC-β	phospholipase C-β
PLD2	phospholipase D2
PVDF	polyvinylidene fluoride
rCBF	regional cerebral blood flow
RHA	right hemisphere area
RIA	right hemisphere infarct area
ROS	reactive oxygen species

SEM	standard error of the mean
sGC	soluble guanylate cyclase
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
TBS-T	tris buffered saline containing 0.1 % tween 20
TEA	tetraethylammonium
VCAM-1	vascular cell adhesion molecule 1

CHAPTER 1

GENERAL INTRODUCTION

The Importance of the Cerebral Circulation

The brain is a highly specialised organ that is central to regulation of all bodily functions. The brain has a minimal reserve of energy and therefore requires a constant supply of oxygen and glucose in order to maintain function. Importantly, the brain is particularly susceptible to ischaemia compared with other organs, such as the liver, which are more resistant to ischaemic damage. Indeed, even a very brief ischaemic period can result in irreversible damage to neuronal tissue. It is therefore not surprising that the cerebral circulation is a highly specialised vascular bed with a number of unique physiological mechanisms designed to ensure adequate blood flow to the brain over a wide range of external and internal conditions. To effectively treat and possibly prevent diseases affecting the cerebral circulation it is important that we better understand the (patho)physiological mechanisms that regulate cerebral artery function during health and disease.

The cerebral circulation possesses a number of developmental features (e.g. cerebrovascular autoregulation and functional hyperaemia) that ensures adequate blood flow to the underlying brain parenchyma. Arterial blood pressure fluctuates widely during daily activity. As such, the cerebral circulation has developed extremely effective autoregulation in order to protect the brain from possible damage due to large variations in systemic blood pressure. Specifically, this involves cerebral artery constriction when blood pressure is raised and dilatation when blood pressure is lowered, such that cerebral blood flow remains relatively stable. One of the most important and unique features of cerebral arteries is the ability to increase cerebral blood flow to match the metabolic demands associated with increased neural activity, termed functional hyperaemia or neurovascular coupling. Neural activity increases the need of brain cells for oxygen and nutrients, as well as the removal of potentially toxic by-products of metabolism by the circulating blood. During increased neural activity, vasoactive agents are released from endothelial cells resulting in dilatation of local and upstream cerebral arteries, thus increasing blood flow to that region of the brain. Numerous agents have been suggested to mediate functional hyperaemia, including products of metabolic activity (e.g. adenosine) (Dirnagl *et al.*, 1994; Meno *et al.*, 2001), nitric oxide (NO·) (Iadecola *et al.*, 1995; Liu *et al.*, 2008) and cyclooxygenase (COX) -2 products (Niwa *et al.*, 2000). Furthermore, there is a growing body

of evidence to suggest that neurons, glia and cerebral blood vessels act as an integrated unit (the neurovascular unit) and play a crucial role in the regulation of functional hyperaemia.

Role of Nitric Oxide in the Regulation of Cerebrovascular Tone

It is well established that endothelium-derived vasoactive factors are crucial regulators of cerebral artery tone. Once released from the endothelium, these vasoactive factors cross the sub-endothelial space and ultimately cause vasodilatation or vasoconstriction. Numerous vasoactive substances have been discovered including the gaseous molecule NO·. In 1980, Furchgott and Zawadzki discovered an endothelium-derived substance that elicited relaxation of rabbit aortae (Furchgott *et al.*, 1980). Subsequently, Palmer and colleagues determined that this endothelium-derived relaxing factor (EDRF) was NO· (Palmer *et al.*, 1987). Since its discovery as an EDRF in the aorta, NO· has been extensively studied and it has become apparent that NO· is central to the regulation of vascular tone in all vascular beds.

NO· can be generated by a family of enzymes known as NO· synthases (NOS). Three isoforms of NOS have been identified, namely inducible NOS (iNOS or NOS1), neuronal NOS (nNOS or NOS2) and endothelial NOS (eNOS or NOS3). iNOS is an inducible, Ca^{2+}-independent enzyme which generates large amounts of NO· upon its activation (Alderton *et al.*, 2001; Michel *et al.*, 1997). In contrast, eNOS and nNOS generate small amounts of NO· constitutively and their activity is Ca^{2+}-dependent (Alderton *et al.*, 2001; Michel *et al.*, 1997). eNOS and nNOS are both constitutively expressed in blood vessels, whereas iNOS is not expressed under normal conditions; however, its expression may be induced in the vessel wall in response to a variety of inflammatory stimuli in a number of disease states (Daneshtalab *et al.*, 2010; Panayiotou *et al.*, 2010; Perrotta *et al.*, 2010). All NOS isoforms catalyze the reaction between L-arginine and molecular oxygen via a two step process involving the generation of the unstable intermediate *N*-hydroxy-L-arginine (NHA) before ultimately producing NO· and L-citrulline (Andrew *et al.*, 1999; Moncada *et al.*, 1991). This process involves the presence of nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), and flavin mononucleotide. Once generated by NOS, NO· can rapidly diffuse to vascular smooth muscle cells where its main target is the cytosolic

enzyme, soluble guanylate cyclase (sGC; see Figure 1). Upon activation, sGC catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which serves as an important second messenger, mediating many of the vascular effects of NO·, cGMP has a number of downstream targets including cGMP-dependent protein kinases (cGKs), phosphodiesterases and cGMP-modulated cation channels (Conti *et al.*, 2007; Feil *et al.*, 2003). Activation of cGK's and cGMP-modulated cation channels can lead to a decrease in intracellular Ca^{2+} and thus cause vasodilatation.

A wealth of studies have shown that activation of eNOS and/or the sGC-cGMP pathway directly leads to cerebral vasodilatation (Bai *et al.*, 2004; Didion *et al.*, 2001; Sobey *et al.*, 1997b). Furthermore, inhibitors of NOS cause constriction of cerebral blood vessels and decrease cerebral blood flow in a number of animal species (Cipolla *et al.*, 2008; Dietrich *et al.*, 1994; Faraci, 1990; Iadecola *et al.*, 1995; McPherson *et al.*, 1995; Yamashiro *et al.*, 2010), indicating that constitutive levels of NO· in the cerebral vasculature are sufficient to influence tone and thus cerebral blood flow. In addition to its effects on cerebrovascular tone, NO· has been reported to play a pivotal role in the regulation of cerebral artery structure (Baumbach *et al.*, 2004). Specifically, in mice, genetic deletion of eNOS or treatment with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), leads to hypertrophy of cerebral arteries (Baumbach *et al.*, 2004). Furthermore, NO· has been demonstrated to inhibit the aggregation of platelets (Bermejo *et al.*, 2005; Mondoro *et al.*, 2001). Thus, it is clear that NO· is central to the regulation of cerebrovascular homeostasis.

Reactive Oxygen Species and Cerebrovascular Tone

Reactive oxygen species (ROS) are a family of highly reactive oxygen-derived molecules that are generated by all cell types within the vasculature. Under normal physiological conditions, ROS levels within the vasculature are tightly regulated, and under these conditions they are believed to serve as important signalling molecules for the regulation of vascular homeostasis. However, if the production of ROS is enhanced or their metabolism impaired, oxidative stress can develop. The parent ROS superoxide (O_2^-) is generated by the one electron reduction of molecular oxygen by numerous oxidases, such as NADPH oxidases, xanthine oxidase, the mitochondrial electron transport chain and cyclooxygenase.

O_2^- is then rapidly metabolised to hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD), namely the copper/zinc-containing cytosolic SOD (SOD1 or Cu-Zn SOD), manganese-containing SOD (SOD2 or Mn-SOD) and the copper/zinc-containing extracellular SOD (SOD3 or EC-SOD). H_2O_2 can then be metabolised to water by catalase or glutathione peroxidases (GPX) (see Figure 1). When O_2^- production is enhanced or its metabolism is decreased in the vessel wall, O_2^- can react with NO·, leading to the generation of the reactive nitrogen species peroxynitrite ($ONOO^-$). Also, H_2O_2 can react with heavy metals such as Fe^{2+} to form the highly reactive hydroxyl radical (OH·) via the Fenton Reaction (see Figure 2).

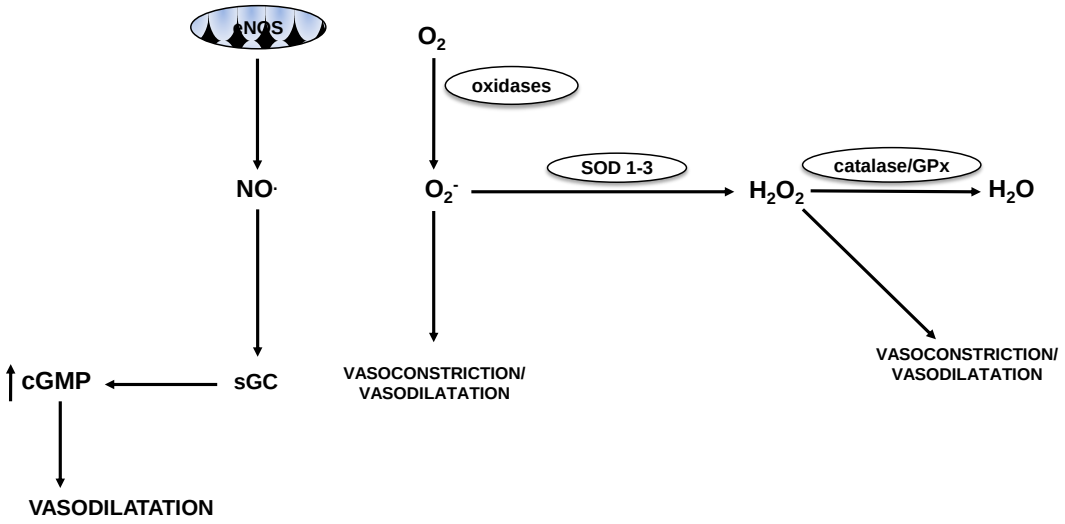


Figure 1. Reactive oxygen species (ROS) as physiological regulators of vascular tone. Within the vasculature, nitric oxide (NO·) is primarily generated by endothelial NO· synthase (eNOS). NO· activates soluble guanylate cyclase (sGC) receptors in the vascular smooth muscle, increasing the concentration of cyclic guanosine monophosphate (cGMP) which results in vasodilatation. Superoxide (O_2^-) is generated by the one electron reduction of molecular oxygen by oxidases, such as NADPH oxidase, xanthine oxidase and cyclooxygenase. During physiological conditions, O_2^- is rapidly metabolised to hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD; SOD1-3), which in turn is metabolised to water by either catalase or glutathione peroxidases (GPx). Both of these ROS may be important signalling molecules within the cerebral vasculature under physiological conditions. Adapted from (Miller *et al.*, 2010).

Superoxide

In cerebral blood vessels from non-diseased animals, O_2^- has been reported to directly modulate vascular tone. Indeed, O_2^- has been shown to elicit dilatation of mouse cerebral arterioles in response to a number of stimuli including bradykinin and NADPH (substrate for NADPH oxidase) (Niwa *et al.*, 2001; Park *et al.*, 2004). Furthermore, it has been demonstrated that O_2^- may mediate relaxation of rabbit cerebral arteries to NADH (NADPH oxidase substrate) (Didion *et al.*, 2002). Moreover, O_2^- generated by exogenously applied xanthine oxidase has been shown to dilate cerebral arterioles from cats (Wei *et al.*, 1996). In

contrast to these studies, it has been demonstrated that O_2^- mediates contraction of canine and rabbit cerebral arteries to the calcium ionophore A23187 and NADH, respectively (Cosentino *et al.*, 1994; Didion *et al.*, 2002). It has been reported that O_2^- elicits relaxation of cerebral arteries via the activation of calcium-activated potassium channels (Didion *et al.*, 2002). Specifically, O_2^--mediated vasorelaxation is attenuated in the presence of the calcium-activated potassium channel blocker tetraethylammonium (TEA) (Didion *et al.*, 2002; Wei *et al.*, 1996). The mechanism by which constriction of cerebral arteries occurs is currently not completely understood. However, it has been postulated that O_2^- may elicit vasoconstriction by either inhibition of calcium-activated potassium channels, or by activation of other vasoconstrictor pathways that have yet to be identified (Didion *et al.*, 2002). It is also unclear why O_2^- has opposing effects on cerebral artery tone, however, one possibility could be that the effects of O_2^- are concentration-dependent, with relaxation occurring at low concentrations and contraction at higher concentrations (Miller *et al.*, 2006a). For example, at low concentrations, both NADPH and NADH (NADPH oxidase substrates) elicit relaxation (Didion *et al.*, 2002), whereas at higher substrate concentrations, O_2^- elicits contraction of rabbit cerebral arteries (Didion *et al.*, 2002).

If the production of O_2^- is enhanced or its metabolism impaired, the major consequence is the reaction of O_2^- with NO·, which is three times faster than the enzymatic dismutation of O_2^- by SOD (Thomson *et al.*, 1995) (see Figure 2). Thus, when O_2^- levels are augmented, it is possible that O_2^- increases cerebrovascular tone by diminishing the bioavailability of NO·. Indeed, clinical and experimental evidence suggests that oxidative inactivation of NO· may be an important underlying mechanism of impaired NO·-dependent responses associated with a number of 'high risk' cardiovascular disease states and cerebrovascular disease. For example, numerous studies have found that scavengers of O_2^- improve NO·-dependent dilatation of both intracranial and extracranial cerebral vessels in numerous animal models of disease, such as hypertension (Didion *et al.*, 2009; Girouard *et al.*, 2006; Schrader *et al.*, 2007), hypercholesterolaemia (d'Uscio *et al.*, 2001; Kitayama *et al.*, 2007; Matsumoto *et al.*, 2003) and ischaemic stroke (Nelson *et al.*, 1992; Xie *et al.*, 2005). In addition to its effects on NO· bioavailability, evidence from the systemic circulation suggests that O_2^- might impair downstream NO· signalling by decreasing the activity and expression of sGC (Gerassimou *et*

al., 2007; Priviero *et al.*, 2009), through mechanisms involving a reduction in the stability of mRNA for sGC (Gerassimou *et al.*, 2007). However, it is unclear if this occurs in cerebral arteries.

Hydrogen Peroxide

Due to its longer half life and ability to diffuse through cell membranes, H_2O_2 may be a particularly important ROS signalling molecule for the modulation of vascular function during health and disease. The overall effects of H_2O_2 on cerebrovascular tone are complex and appear to differ between species. Indeed, H_2O_2 has been reported to elicit both dilatation and constriction of cerebral arteries. For example, studies have found that H_2O_2 mediates dilatation of mouse and rat cerebral arteries to arachidonic acid (Modrick *et al.*, 2009), bradykinin (Sobey *et al.*, 1997a) and flow (Drouin *et al.*, 2009; Paravicini *et al.*, 2004; Paravicini *et al.*, 2006). Furthermore, NADPH oxidase-derived H_2O_2 offsets contraction of the rat basilar artery to angiotensin II (Miller *et al.*, 2005). Moreover, exogenous application of H_2O_2 to the rat basilar artery causes a small contraction followed by sustained relaxation response (Miller *et al.*, 2005). In contrast, exogenous H_2O_2 has been reported to mediate contraction of cerebral arteries from monkeys (Toda *et al.*, 1992). With respect to its potential vasodilatory effects, evidence suggests that that H_2O_2-mediated vasodilatation involves the opening of calcium-activated potassium channels in rats (Sobey *et al.*, 1997a), whereas ATP-sensitive potassium channels have been implicated in mediating H_2O_2-induced dilatation of feline cerebral arteries (Wei *et al.*, 1996). In contrast, the mechanisms mediating H_2O_2-induced vasoconstriction of cerebral arteries are less well understood and warrant further investigation. Further studies are needed to determine the overall effects of H_2O_2 on cerebral vascular tone in humans.

In addition to its effects on cerebrovascular tone, high concentrations of exogenous H_2O_2 have been shown to induce apoptosis of cerebral vascular cells (Li *et al.*, 2003a), which would conceivably result in impaired cerebral artery reactivity. Furthermore, experimental evidence suggests that H_2O_2 may increase vascular tone by impairing NO· function following its conversion to OH· via the Fe^{2+} catalyzed fenton reaction. Specifically, it has been shown that impaired NO·-induced vasodilatation in feline cerebral arteries following treatment

with exogenous H_2O_2 is prevented by the iron chelator, deferoxamine (Wei *et al.*, 1990) (see Figure 2). More recently, H_2O_2 has been shown to mediate endothelial cell contraction, leading to blood brain barrier dysfunction and oedema formation following cerebral ischaemia-reperfusion (Kahles *et al.*, 2007).

Peroxynitrite

$ONOO^-$ is the product of the reaction of O_2^- with NO·. In healthy blood vessels, the production and metabolism of O_2^- is tightly regulated and thus it is unlikely that $ONOO^-$ is generated under physiological conditions. However, $ONOO^-$ is generated within the vasculature during disease and has been implicated as an important mediator of vascular dysfunction. For example, has been reported that $ONOO^-$ can impair the regulation of cerebrovascular function by causing nitrosative damage to key regulatory proteins, such as F-actin, leading to impaired contractility of cerebral blood vessels (DeWitt *et al.*, 2001; Girouard *et al.*, 2007; Maneen *et al.*, 2007). In systemic arteries, it has been shown that $ONOO^-$ can oxidise BH_4 (Chen *et al.*, 2010; Landmesser *et al.*, 2003; Zou *et al.*, 2002), as well as the zinc-thiolate complex in eNOS (Zou *et al.*, 2002), both of which can lead to eNOS uncoupling and the production of O_2^- instead of NO· (see Figure 2). $ONOO^-$ may also impair NO· signalling via oxidation of the haem group of sGC, converting it from its Fe^{2+}-bound to is Fe^{3+}-bound NO·-insensitive state (Stasch *et al.*, 2006) (see Figure 2). However, to the best of our knowledge, no study has examined whether $ONOO^-$ promotes uncoupling of eNOS and/or oxidises sGC in the cerebral circulation.

In addition to its potential effects on cerebral vascular tone through impairing NO· signalling, studies suggest that $ONOO^-$ may have direct effects on cerebral vascular tone. Indeed, exogenous $ONOO^-$ has also been shown to dilate feline and canine cerebral arteries (Li *et al.*, 2004; Wei *et al.*, 1996). Multiple mechanisms have been proposed to mediate relaxation responses to $ONOO^-$, including activation of potassium channels, membrane hyperpolarisation and elevation of cGMP levels (Li *et al.*, 2004; Wei *et al.*, 1996). In contrast to these findings, $ONOO^-$ has also been shown to constrict cerebral arteries *in vitro* via the attenuation of basal activity of calcium-activated potassium channels (Brzezinska *et al.*, 2000; Elliott *et al.*, 1998).

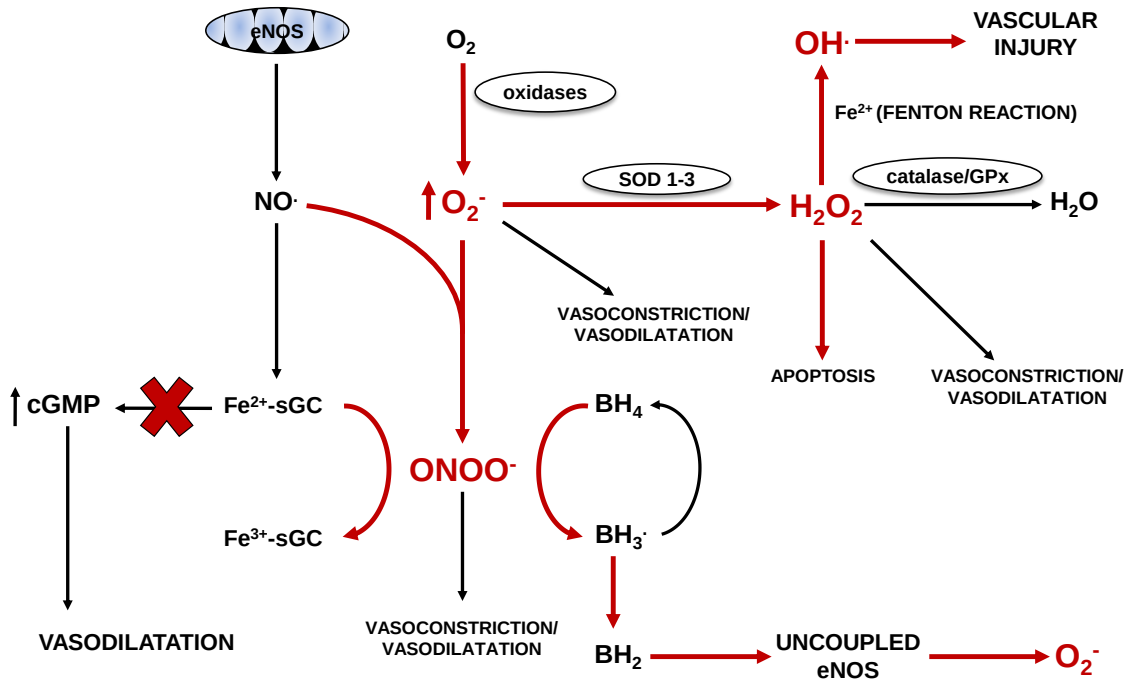


Figure 2. Reactive oxygen species (ROS) and nitric oxide (NO·) during disease. During physiological conditions, the levels if ROS are tightly regulated and ROS may serve as mediators of cerebral artery constriction of dilatation. However, if the production of ROS is enhanced and/or its metabolism impaired, oxidative stress can ensue. Excessive levels of superoxide (O_2^-) may scavenge nitric oxide (NO·), reducing its bioavailability and thus, effects on vascular homeostasis. This also results in the formation of peroxynitrite ($ONOO^-$), which can oxidise the haem group of soluble guanylate cyclase (sGC), rendering it insensitive to NO·. Furthermore, $ONOO^-$ can oxidise the endothelial NO· synthase (eNOS) cofactor tetrahydrobiopterin (BH_4), resulting in eNOS uncoupling and the subsequent production of O_2^- instead of NO·. Hydrogen peroxide (H_2O_2) may cause apoptosis and also induce vascular injury via its conversion to hydroxyl radical (OH·). BH_3, trihydrobiopterin; BH_2 7,8-dihydrobiopterin. Adapted from (Miller *et al.*, 2010).

Sources of Reactive Oxygen Species within the Vasculature

A number of enzymes within vascular cells are capable of generating ROS, including xanthine oxidase (Kinugawa *et al.*, 2005), cyclooxygenases (Niwa *et al.*, 2001), uncoupled

NOS (Dikalova *et al.*, 2010; Landmesser *et al.*, 2003) and the mitochondrial electron transport chain (Narayanan *et al.*, 2010). However, all of these enzyme systems generated ROS as either a by-product of normal enzyme activity or when in dysfunctional state. By contrast, NADPH oxidases are believed to be the only known family of enzymes whose primary role is the generation of ROS. Thus, NADPH oxidases have been identified as likely candidates for deliberate ROS production required for signalling during normal physiology, and for the excessive ROS production that is thought to lead to oxidative stress in various vascular diseases. Currently, five isoforms of NADPH oxidase have been identified and are named after the identity of the 'Nox' catalytic subunit: Nox1-, Nox2-, Nox3-, Nox4- and Nox5- containing NADPH oxidases. In general, all NADPH oxidases generate O_2^- by transferring electrons from the substrate, NADPH, via the Nox catalytic subunit to molecular oxygen. Nox1-, Nox2-, Nox4- and Nox5-containing isoforms are all expressed within vascular cells, however, to date only the Nox1-, Nox2- and Nox4-containing isoforms have been identified in the cerebral vasculature (Ago *et al.*, 2005; Miller *et al.*, 2007b). In addition to containing different Nox catalytic subunit, each isoform appears to require different regulatory cytosolic subunits for oxidase activity (see below).

Nox2-containing NADPH Oxidase

Structure of Nox2-NADPH Oxidase

The structure and function of Nox2-NADPH oxidase (originally known as gp91phox) was initially characterized in neutrophils, where it plays a role in immunological host defence. Nox2-NADPH oxidase consists of two membrane-bound subunits (Nox2 and p22phox), up to three cytosolic subunits (p47phox [organiser subunit], p67phox [activator subunit] and potentially p40phox) and the small G-proteins Rac (see Figure 3). The catalytic domain resides in Nox2, which contains all of the necessary components to facilitate the transfer of electrons from the substrate, NADPH to molecular oxygen. Specifically, the catalytic domain of Nox2 possesses two haem containing groups and a FAD group, as well as a binding site for NADPH (Cheng *et al.*, 2001; Taylor *et al.*, 1993). The p22phox subunit forms a heterodimer with Nox2 and contains a proline rich region that facilitates the binding of p47phox with the membrane bound subunits (Groemping *et al.*, 2003). As such, p22phox plays a crucial role in regulating Nox2 activity (Ambasta et al, 2004). In resting cells, Nox2 is dormant, and its

activation requires the interaction of Nox2/p22phox with the regulatory cytosolic subunits p47phox and p67phox. Under resting conditions, p47phox is not associated with the Nox2/p22phox complex due to the presence of an autoinhibitory domain which prevents the interaction of p47phox with the proline rich region of p22phox (Groemping *et al.*, 2003). Translocation of p47phox to the membrane bound subunits is dependent on protein kinase C (PKC)-dependent phosphorylation of serine residues on p47phox (Fontayne *et al.*, 2002), which results in a change in conformation and displacement of the autoinhibitory region (Groemping *et al.*, 2003). p67phox binds to p47phox and this complex translocates to the membrane where two SH3 domains on p47phox bind to the proline rich region of p22phox (Groemping *et al.*, 2003; Nobuhisa *et al.*, 2006). In addition, p47phox has also been shown to bind multiple sites on Nox2, which is believed to be crucial for the final assembly of the active oxidase (DeLeo *et al.*, 1995). It has been suggested that the binding of Rac induces a conformation change in p67phox, which in turn promotes its binding to Nox2 (Sarfstein *et al.*, 2004), which leads to a conformation change and electron flow through the Nox2 subunit (Nisimoto *et al.*, 1999). Much less is known regarding the role of p40phox in the activation of Nox2-NADPH. However, in coronary endothelial cells isolated from p47phox-deficient mice, it has been suggested that p40phox may act as an alternative organiser subunit in the absence of p47phox (Fan *et al.*, 2009). Currently, it remains to be determined if p40phox is necessary for Nox2-NADPH oxidase activation in the cerebral circulation. The latter point notwithstanding, once the Nox2-NADPH oxidase enzyme complex is assembled, electrons can then be transferred from NADPH to FAD and then to the haem groups and finally to molecular oxygen, thus generating O_2^-.

Expression of Vascular Nox2-NADPH Oxidase

Our understanding of the expression profile of NADPH oxidase isoforms in cerebral arteries lags behind that of systemic arteries. However, it has been revealed that mRNA for the Nox2 catalytic subunit, as well as p22phox and the cytosolic subunit p47phox, are expressed in the rat basilar artery (Paravicini *et al.*, 2004). Furthermore, Nox2 protein has been found to be expressed in endothelial and adventitial cells of cerebral arteries from mice (Kazama *et al.*, 2004; Miller *et al.*, 2009). Furthermore, Kazama *et al*. found that Nox2 is expressed on intracellular membranes, rather than at the cell membrane (Kazama *et al.*, 2004).

Activation of Nox2-NADPH Oxidase

As discussed above, the key regulatory components of Nox2-NADPH are located in the cytosol at rest, suggesting that Nox2-NADPH oxidase may not be basally active. Indeed, it has been demonstrated that Nox2-NADPH oxidase does not contribute to basal O_2^- production in mouse intracranial cerebral arteries (Miller *et al.*, 2009). However, Nox2-NADPH oxidase has been reported to contribute to NADPH-stimulated O_2^- production in mouse intracranial cerebral arteries (see *Cerebrovascular NADPH Oxidase Under Normal Physiological Conditions*) (Miller *et al.*, 2009). A number of stimuli can increase the activity of Nox2-NADPH oxidase, however, angiotensin II is regarded as one of the most important and well studied physiological activators of Nox2-NADPH oxidase. Indeed, numerous studies have reported that angiotensin II increases O_2^- production by NADPH oxidase in both intact arteries and cultured vascular cells (Capone *et al.*, 2010b; Choi *et al.*, 2008; Didion *et al.*, 2003; Girouard *et al.*, 2006; Griendling *et al.*, 1994; Kazama *et al.*, 2004; Miller *et al.*, 2005), through activation of the angiotensin type 1 receptor (AT_1 receptor) (Choi *et al.*, 2008; Girouard *et al.*, 2006). In cerebral arteries, evidence indicates that angiotensin II-stimulated O_2^- production is primarily derived from Nox2-NADPH oxidase (Girouard *et al.*, 2006; Girouard *et al.*, 2007; Kazama *et al.*, 2004). Specifically, it has been demonstrated that ROS production in response to angiotensin II is substantially attenuated in cerebral arteries from Nox2-deficient mice (Girouard *et al.*, 2006; Kazama *et al.*, 2004).

It remains to be fully understood how angiotensin II increases O_2^- production by Nox2-NADPH oxidase in the cerebral circulation. In non-cerebrovascular cells, activation of the AT_1 receptor by angiotensin II predominantly results in a phospholipase C-β (PLC-β) –mediated increase in inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) and subsequent activation of PKC (Choi *et al.*, 2008) (see Figure 3). Angiotensin II also activates phosphatidylinositol 3-kinase (PI3-K), resulting in Rac activation which is critical for Nox2-NADPH oxidase activity (Choi *et al.*, 2008). As discussed above, phosphorylation of p47phox by PKC results in the assembly of the Nox2-NADPH oxidase complex and O_2^- generation. It has also been demonstrated that phospholipase D2 (PLD2) partially contributes to angiotensin II-mediated activation of Nox2-NADPH oxidase in cultured cells, possibly by

supplementing DAG levels, which is necessary for sustained PKC activation (Choi *et al.*, 2008) (see Figure 3).

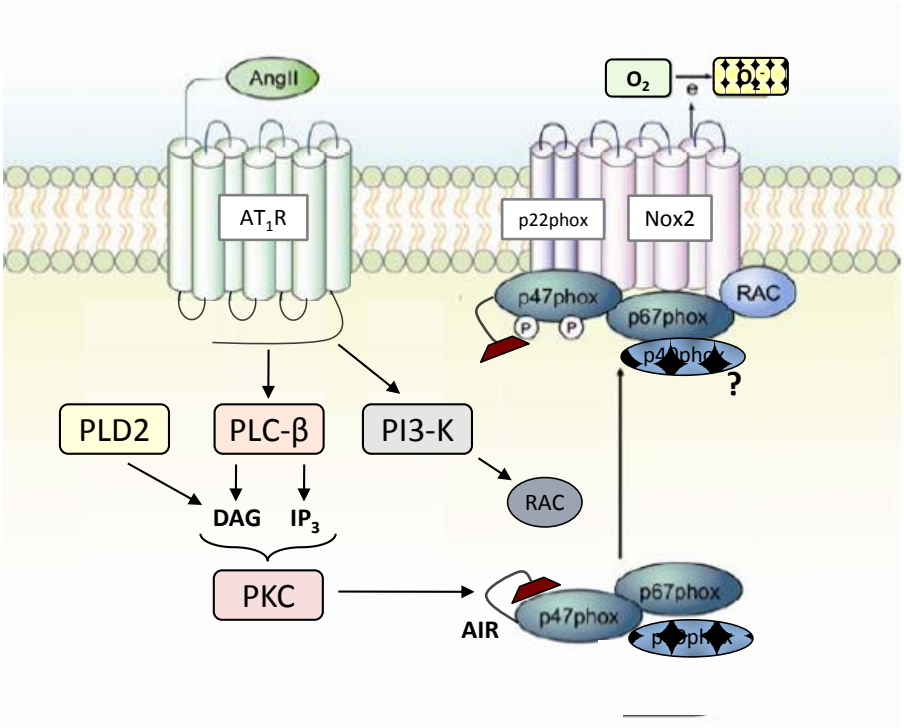


Figure 3. Structure and activation of Nox2-NADPH oxidase. The Nox2-containing isoform of NADPH oxidase consist of two membrane-bound subunits, Nox2 and p22phox, up to three cytosolic subunits, p47phox, p67phox and p40phox, as well as the G-protein Rac. A number of stimuli can increase the activity of Nox2-NADPH oxidase, including angiotensin II (AngII). Activation of the AT_1 receptor (AT_1R) by angiotensin II results in activation of phospholipase C-β (PLC-β) and an increase in the intracellular concentration of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which ultimately activates protein kinase C (PKC). Phosphatidylinositol 3-kinase (PI3-K) is also activated and promotes Rac activation. PKC phosphorylates p47phox revealing the p22phox binding site. p47phox, p67phox and Rac translocate to the membrane where they bind to the Nox2/p22phox complex, forming the active complex of the enzyme. p40phox may or may not be required for Nox2-NADPH oxidase activity in cerebral arteries. This last point notwithstanding, electrons are transferred from the substrate NADPH via the Nox catalytic subunit to molecular oxygen, generating superoxide (O_2^-). Adapted from (Choi *et al.*, 2008).

Nox1- & Nox4- NADPH Oxidases

The first homologue of Nox2-NADPH oxidase, Nox1-NADPH oxidase (originally Mox1) was discovered over 10 years ago (Suh *et al.*, 1999). Nox1-NADPH oxidase is structurally similar to Nox2-NADPH oxidase but appears to utilise different regulatory cytosolic subunits for its activation. Like the Nox2 subunit, the Nox1 subunit contains two haem groups, FAD and NADPH binding sites, and forms a heterodimer with p22phox in cell membrane (Ambasta *et al.*, 2004; Kawahara *et al.*, 2005). Nox1-NADPH oxidase is activated by NoxO1 (Nox organiser 1) and NoxA1 (Nox activator 1), which are homologues of p47phox and p67phox, respectively (Banfi *et al.*, 2003) (see Figure 4). Nox1-NADPH may be able to be activated by p47phox and p67phox (Banfi *et al.*, 2003). However, the interaction of Nox1 with NoxO1/NoxA1 is more effective than its interaction with p46phox/p67phox (Banfi *et al.*, 2003). It was recently shown that preventing Rac activation by inhibiting PI3-K only attenuates Nox1-NADPH oxidase activity by approximately 20 % (Choi *et al.*, 2008), suggesting that it may not be critical for Nox1-NADPH oxidase activity. Unlike p47phox, NoxO1 lacks an autoinhibitory domain and it has been hypothesised that the Nox1-NADPH oxidase might be basally active (Banfi *et al.*, 2003). However, recent studies using Nox1-deficient mice suggest that Nox1-NADPH oxidase does not contribute to basal O_2^- production in mouse cerebral arteries (Jackman *et al.*, 2009b). However, angiotensin II-stimulated O_2^- production is attenuated in cerebral arteries from Nox1-deficient mice (Jackman *et al.*, 2009b), suggesting that Nox1-NADPH oxidase contributes to angiotensin II stimulated ROS production in the cerebral circulation. Interestingly, it has been reported that AT_1 receptors are located intracellularly in aortic smooth muscle cells from Nox1-deficient mice, (Basset *et al.*, 2009), suggesting that Nox1-derived ROS may regulate cell surface expression of AT_1 receptors. While it remains to be established if this occurs in cerebral vessels, it is conceivable that the attenuation in angiotensin II-stimulated O_2^- production found in cerebral arteries from Nox1-deficient mice may actually be due to impaired trafficking of the AT_1 receptor to the cell membrane and thus, reduced activation by angiotensin II.

Nox4-NADPH oxidase was discovered in the kidney (originally Renox) where is was proposed to serve as an oxygen sensing enzyme (Geiszt *et al.*, 2000). Similar to Nox1- and Nox2-

NADPH oxidase, the Nox4 catalytic domain contains all of the required components for oxidase activity and forms a heterodimer with p22phox (Ambasta *et al.*, 2004; Martyn *et al.*, 2006). However, one unique feature of Nox4-NADPH oxidase is that it may not require cytosolic subunits or Rac for oxidase activity and may be basally active (Martyn *et al.*, 2006) (see Figure 4). Also, evidence suggests that Nox4-NADPH oxidase may directly generate H_2O_2 in addition to O_2^- (Martyn *et al.*, 2006).

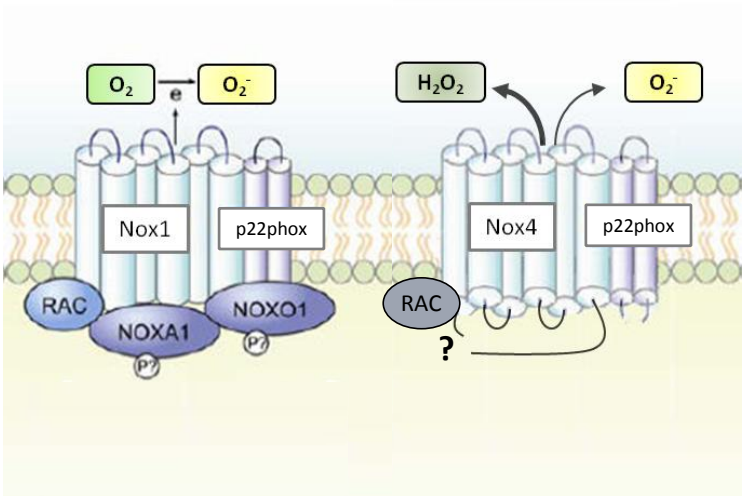


Figure 4. Structure of Nox1- and Nox4 -NADPH oxidase. The Nox1-containing isoform of NADPH oxidase consist of two membrane-bound subunits, Nox1 and p22phox, two other regulatory subunits, NoxO1 and NoxA1 (which are homologues of p47phox and p67phox) and the G-protein Rac. NoxO1 lacks the autoinhibitory domain of its homologue p47phox and as such, it has been postulated that Nox1-NADPH oxidase may be constitutively assembled. The activity of Nox1-NADPH oxidase can be increased by stimuli such as angiotensin II, however this may be due to impaired AT_1 receptor trafficking in the absence of Nox1-NADPH oxidase (see text). Nox4-NADPH oxidase may not require cytosolic subunits for activity and may be basally active. Nox4-NADPH oxidase may also directly generate hydrogen peroxide (H_2O_2) rather than superoxide (O_2^-). Similar to all NADPH oxidase isoforms, once activated, electrons are transferred from the substrate, NADPH, via the Nox catalytic subunit, generating reactive oxygen species. Adapted from (Choi *et al.*, 2008).

Expression of Nox1 & Nox4- NADPH Oxidases

Messenger RNA for the Nox1 and Nox4 catalytic subunits are expressed in rat basilar arteries (Ago *et al.*, 2005; Paravicini *et al.*, 2004). Specifically, Nox1 and Nox4 mRNA are expressed predominantly in basilar artery endothelial cells and to a lesser extent vascular smooth muscle cells (Ago *et al.*, 2005). More recently, Nox1 and Nox4 expression at the protein level has been demonstrated in rat basilar arteries (Miller *et al.*, 2007b).

Regional Differences in NADPH Oxidase Activity and Expression

Recently it has become evident that stark differences exist between cerebral and non-cerebral arteries with respect to the activity of NADPH oxidase under physiological conditions. Specifically, NADPH-induced O_2^- production is up to 100-fold higher in intracranial cerebral arteries versus a range of systemic arteries such as the common carotid, aorta and mesenteric artery in rats, mice, rabbits and pigs (Miller *et al.*, 2005; Miller *et al.*, 2009). Furthermore, angiotensin II-stimulated O_2^- production by rat basilar arteries from rats is also profoundly greater than systemic arteries (Miller *et al.*, 2005). Nox1 (mRNA), Nox2 (mRNA and protein) and Nox4 (mRNA and protein) expression is higher in rat and mouse cerebral arteries compared with systemic arteries (Ago *et al.*, 2005; Miller *et al.*, 2005). Thus, greater relative expression of NADPH oxidase isoforms may account for the higher NADPH oxidase activity in cerebral versus systemic arteries (Miller *et al.*, 2005). Indeed, in Nox2-deficient mice, NADPH-stimulated O_2^- production by cerebral arteries but not systemic arteries (aorta and carotid) is lower than levels generated by arteries from wild-type mice (Miller *et al.*, 2009). Taken together, these findings raise the possibility that NADPH oxidases might be a particular important source of ROS in the cerebral circulation during health and disease.

Cerebrovascular NADPH Oxidase Under Normal Physiological Conditions

Numerous laboratories have reported that NADPH oxidases are expressed and active in cerebral arteries from non-diseased animals (Ago *et al.*, 2005; Didion *et al.*, 2002; Miller *et al.*, 2005; Miller *et al.*, 2009; Miller *et al.*, 2007b; Paravicini *et al.*, 2006; Park *et al.*, 2004).

However, using genetically deficient mice it has been demonstrated that neither Nox1- nor Nox2-NADPH oxidase contribute to basal O_2^- production by cerebral arteries (Jackman *et al.*, 2009b; Miller *et al.*, 2009). Recent work from our lab has demonstrated that basal O_2^- production by mouse cerebral arteries is attenuated by the flavin antagonist/NADPH oxidase inhibitor diphenyleneiodonium (DPI) (Ku, Sobey and Miller, unpublished observations), suggesting that an NADPH oxidase isoform or another flavin-containing enzyme may contribute to basal O_2^- production in the cerebral vasculature. As discussed, Nox4-NADPH oxidase may be active under basal conditions in non-cerebral vascular cells (Martyn *et al.*, 2006). Moreover, Nox4 protein expression is higher in cerebral arteries compared with systemic arteries (Miller *et al.*, 2005). Thus, while future work is needed it is conceivable that Nox4-containing NADPH oxidase may be an important source of basal O_2^- in cerebral arteries. In contrast with basal O_2^- production, it has been demonstrated that Nox2-NADPH oxidase contributes to NADPH-stimulated O_2^- production by cerebral arteries (Miller *et al.*, 2009). Thus, evidence would suggest that Nox2-NADPH oxidase may be expressed at relatively low levels during physiological conditions but can be activated when required.

As discussed above, ROS have been shown to modulate cerebrovascular tone in healthy animals (see *Reactive Oxygen Species and Cerebrovascular Tone*). For example, application of NADPH or NADH has been reported to elicit profound cerebrovascular dilatation in rabbits, rats and mice (Didion *et al.*, 2002; Miller *et al.*, 2005; Miller *et al.*, 2007b; Paravicini *et al.*, 2004; Park *et al.*, 2004). As discussed previously, ROS-mediated modulation of vascular tone is complex and the identity of the ROS that mediates NAD(P)H-induced dilatation may be species and/or concentration dependent. NADPH-induced dilatation is inhibited by DPI or the NADPH oxidase assembly inhibitor apocynin and is attenuated in mice genetically deficient in Nox2 (Miller *et al.*, 2005; Paravicini *et al.*, 2004; Park *et al.*, 2004). NADPH oxidase-derived ROS are believed to mediate cerebrovascular dilatation in response to flow (Paravicini *et al.*, 2006), suggesting that NADPH oxidases may indeed modulate vascular tone *in vivo*. Furthermore, NADPH oxidase-derived H_2O_2 has been shown to offset contractions to angiotensin II *in vitro* (Miller et al., 2005). Taken together, these findings point towards a potential role for NADPH oxidase-derived ROS in cerebral

vasodilator. However, future work is needed to define the roles of NADPH oxidase-derived ROS in regulating cerebral vascular tone in other animals and, in particular, humans.

NADPH Oxidases and Cerebrovascular Tone During Disease

During physiological conditions, the production and metabolism of ROS is tightly regulated. However, if ROS production is enhanced and/or ROS metabolism is impaired, oxidative stress can develop. Within the cerebral circulation, oxidative stress is associated with a number of diseases and high risk cardiovascular states, such as hypertension (Capone *et al.*, 2010b; Girouard *et al.*, 2006) and hypercholesterolaemia (Kitayama *et al.*, 2007) and diseases such as stroke (Kunz *et al.*, 2006; McCann *et al.*, 2008). NADPH oxidases have been identified as prime candidates for the excessive ROS production that is thought to lead to oxidative stress in various vascular diseases. Furthermore, in cerebral arteries, many of the deleterious effects of oxidative stress have been attributed to excessive production of ROS by the Nox2-containing isoform of NADPH oxidase (Capone *et al.*, 2010b; Girouard *et al.*, 2006; Girouard *et al.*, 2007; Kazama *et al.*, 2004; Park *et al.*, 2007).

Hypertension

Hypertension has profound effects on the cerebral circulation and is a major risk factor for stroke and vascular cognitive impairment (Kivipelto *et al.*, 2001; Lewington *et al.*, 2002; O'Donnell *et al.*, 2010). Within the cerebral circulation, hypertension has been reported to promote cerebrovascular remodelling (Baumbach *et al.*, 2003) and inflammation (Ando *et al.*, 2004), as well as impairing NO·-dependent vasodilatation (Capone *et al.*, 2010a; Girouard *et al.*, 2006; Kazama *et al.*, 2003). One of the key mediators in the pathogenesis of hypertension is the octapeptide angiotensin II. Importantly, NADPH oxidase-derived ROS production is believed to play a central role in the development of angiotensin II-dependent hypertension. In 1996, Rajagopalan *et al*., demonstrated that angiotensin II-induced hypertension was associated with markedly elevated O_2^- production by NADPH oxidase (Rajagopalan *et al.*, 1996). Since this discovery, numerous studies have demonstrated that NADPH oxidase-derived ROS play a central role in the pathogenesis of angiotensin II-induced hypertension (Dikalova *et al.*, 2005; Matsuno *et al.*, 2005; Yogi *et al.*, 2008).

Nox2-NADPH oxidase is believed to mediate many of the deleterious effects of hypertension on the cerebral circulation. Indeed, numerous studies have reported that ROS production is augmented, agonist-induced NO·-dependent dilatation and functional hyperaemia are impaired in cerebral arteries from hypertensive animals (Capone *et al.*, 2010b; Girouard *et al.*, 2008; Girouard *et al.*, 2006; Kazama *et al.*, 2004; Kazama *et al.*, 2003). Moreover, such changes are absent in Nox2-deficient mice or in wild type mice treated with O_2^- scavengers, suggesting that the augmented O_2^- production may be acutely scavenging NO· during disease (Capone *et al.*, 2010b; Girouard *et al.*, 2007; Kazama *et al.*, 2004). Interestingly, a recent study has reported that angiotensin II-induced dysfunction of cerebral arteries occurs independently of blood pressure elevation. Specifically, in mice treated with a slow-pressor dose of angiotensin II, ROS production was elevated in cerebral arterioles and NO·-dependent dilatations (agonist-induced and functional hyperaemia response) were impaired prior to the elevations in systolic blood pressure (Capone *et al.*, 2010b). Moreover, the impaired NO·-dependent vasodilatation was reversed by a scavenger of O_2^- and the NADPH oxidase inhibitor, gp91ds-tat (Capone *et al.*, 2010b). These findings suggest that prior to the development of hypertension, angiotensin II causes cerebral vascular dysfunction via Nox2-NADPH oxidase-derived ROS. Furthermore, this raises the possibility that the cerebral circulation is sensitive to the effects of hypertension, which may be due to the greater expression of Nox2-NADPH oxidase in cerebral arteries compared with systemic arteries (Miller *et al.*, 2009). Therefore, angiotensin II causes increased ROS production and impaired NO·-dependent vasodilatation in the cerebral circulation in the absence and presence of changes in blood pressure. In vivo, this would manifest as impaired functional hyperaemia and may lead to hypoperfusion of the brain, which would conceivably lead to impaired cognitive function.

As discussed in previously, evidence to date points towards a potential role for NADPH oxidases in cerebral vasodilator responses under physiological conditions (Didion *et al.*, 2002; Miller *et al.*, 2005; Miller *et al.*, 2007b; Park *et al.*, 2004). Interestingly, it has been postulated that NADPH oxidase-derived ROS may play a beneficial role for cerebral perfusion during disease (Miller *et al.*, 2006a). Indeed, NADPH-induced vasodilatation *in vivo*

is enhanced in chronic hypertension (Paravicini *et al.*, 2004). However, it is currently unknown if this is occurs in all species or if it is unique to the rat cerebral circulation.

Hypercholesterolaemia

Epidemiological studies have reported both positive (Iso *et al.*, 1989; Kannel *et al.*, 1965) and negative (Kagan *et al.*, 1980; Wannamethee *et al.*, 2000) correlations between total cholesterol levels and the incidence of stroke. As such, debate has existed as to whether hypercholesterolaemia is a risk factor for stroke. It has become apparent that one of the main reasons for this has been that investigators have used total cholesterol levels rather than separating serum cholesterol into its two main subtypes (high and low density lipoprotein; HDL and LDL, respectively). Indeed, more recent studies have demonstrated a significant correlation between plasma LDL cholesterol and ischaemic stroke incidence (Amarenco *et al.*, 2006; Imamura *et al.*, 2009).

Interestingly, it has been shown that the development of atherosclerotic lesions in intracranial cerebral arteries occurs at a slower rate and is less severe than atherosclerotic lesion development in systemic arteries of both humans (D'Armiento *et al.*, 2001; Mathur *et al.*, 1963; Napoli *et al.*, 1999; Sadoshima *et al.*, 1980) and animal models of hypercholesterolaemia/atherosclerosis (Didion *et al.*, 2001; Hoekstra *et al.*, 2008; Ito *et al.*, 2001; Kitagawa *et al.*, 1994; Kitayama *et al.*, 2007; Suzuki *et al.*, 2006). These findings suggest that the cerebral circulation may be relatively protected from atherosclerotic lesion development. However, despite the lack of lesions, some (Didion *et al.*, 2001; Kitayama *et al.*, 2007; Rossitch *et al.*, 1991; Shimokawa *et al.*, 1988; Stewart-Lee *et al.*, 1991; Yamashiro *et al.*, 2010), but not all (Kitagawa *et al.*, 1994; Simonsen *et al.*, 1991) studies report that NO·-dependent relaxation responses are impaired in cerebral arteries from hypercholesterolaemic animals. Excessive ROS production has been implicated in the cerebral vascular dysfunction associated with hypercholesterolaemia. Indeed, a recent study found that the SOD mimetic tempol restores NO·-dependent vasodilatation of cerebral arterioles in hypercholesterolaemic mice (Kitayama *et al.*, 2007). Furthermore, evidence suggests that increased O_2^- production by cerebral vascular NADPH oxidases may play a key role in diminished NO· function during hypercholesterolaemia. Indeed, Kitayama et al (2007)

found that NO·-dependent vasodilatation of cerebral arterioles in hypercholesterolaemic mice are restored following treatment with the NAPDH oxidase inhibitor apocynin (Kitayama *et al.*, 2007). However, it is currently unclear which isoform(s) of NADPH oxidase mediates such changes.

Recently, the mechanisms contributing to the vascular dysfunction induced by hypercholesterolaemia has come under scrutiny. In the systemic circulation, the role of the immune system has received particular attention. Specifically, it has been demonstrated that T-lymphocytes may infiltrate the vasculature and be a cause of the impaired NO·-dependent vasodilatation (Ishikawa et al., 2004; Stokes et al., 2007a; Stokes et al., 2007b). However, it is currently unclear if T-lymphocytes mediate the deleterious effects of hypercholesterolaemia in the cerebral circulation.

Cerebral Ischaemia and Reperfusion

Ischaemic stroke is a devastating and potentially life threatening cerebrovascular event. Cerebral ischaemia occurs due to the occlusion of a cerebral artery by an embolus, which may arise in systemic or extracranial cerebral arteries and lodges in a cerebral artery, or by local thrombosis. In 1977, Astrup and colleagues identified two distinct regions develop as a result of the reduction in blood flow (Astrup *et al.*, 1977). In the area of the brain subjected to a severe reduction in blood flow, neurons undergo necrotic cell death, whereas regions where blood flow was constrained, neurons were functionally inactive but still alive (Astrup *et al.*, 1977). This region is known as the penumbra and been intensively investigated over the years as it possibly represents the area of brain tissue that may be salvaged by therapies. The progression of tissue damage following cerebral ischaemia involves a complex series of events, occurring over a period of seconds to days. Investigators have identified a number of contributors to ischaemic brain damage, which are: (1) energy failure and excitotoxicity; (2) oxidative stress; (3) peri-infarct depolarisations; (4) inflammation; and (5) apoptosis (Moskowitz *et al.*, 2010). In addition to causing irreversible damage to the brain parenchyma, it has been extensively demonstrated that cerebrovascular reactivity is profoundly altered following cerebral ischaemia-reperfusion (see below).

Currently, the only approved therapy for ischaemic stroke is tissue plasminogen activator (tPA). tPA catalyses the conversion of plasminogen to plasmin which is the major enzyme responsible for clot breakdown. However, tPA can only be administered to a relatively small percentage of patients that present with ischaemic stroke due to an increased risk of fatal intracranial bleeding. As such, there is a great need to develop new therapies or therapeutic strategies for acute stroke therapy. Experimental evidence indicates that cerebrovascular function is profoundly altered during the initial few hours after the start of reperfusion. Specifically, it has been reported that ROS production is elevated (Kontos *et al.*, 1992; Mori *et al.*, 1999; Nelson *et al.*, 1992) and NO·-dependent relaxation responses are impaired (Clavier *et al.*, 1994; Mayhan *et al.*, 1988; Nelson *et al.*, 1992; Rosenblum *et al.*, 1997) up to two hours after the start of ischaemia. Furthermore, in rats, basal NO· function is impaired 24 h after cerebral ischaemia (Cipolla *et al.*, 2008; Cipolla *et al.*, 2009b; Marrelli *et al.*, 1999). However, conflicting reports exist regarding the role of augmented ROS, specifically O_2^-, production in attenuating NO·-dependent relaxation responses following cerebral ischaemia. Specifically, shortly after the induction of reperfusion (i.e. up to 2 h), ROS scavengers have been shown to improve (Nelson *et al.*, 1992) or have no effect (Rosenblum *et al.*, 1997) on NO·-dependent relaxation responses following ischaemic stroke. The reason(s) for these conflicting reports are unclear and clearly further investigation into the role of vascular ROS in NO· after cerebral ischaemia are warranted. More recently, it has become apparent that augmented O_2^- production persists long after the ischaemic insult and reperfusion. Indeed, NADPH-stimulated O_2^- production is elevated in cerebral arteries up to 72 h after mild cerebral ischaemia in rats (Miller *et al.*, 2006b), suggesting that NADPH oxidases may be a important source of ROS in cerebral arteries following ischaemia and reperfusion. However, the identity of the NADPH oxidase isoform responsible for generating excessive levels of ROS following cerebral ischaemia-reperfusion is currently unknown. Overall, evidence from the aforementioned experimental studies clearly demonstrates that cerebral ischaemia has damaging effects on the function of cerebral arteries. Therefore, it is conceivable that cerebral blood flow to the penumbra may be compromised and this may result in apoptosis of these at risk neurons. Therefore, therapies targeted at improving post ischaemic blood flow may be of benefit.

It is well established that Nox2-NADPH oxidase contributes to poorer neurological outcome following cerebral ischaemia. In 1997, Walder and colleagues identified Nox2-NADPH oxidase as detrimental to neurological outcome following cerebral ischaemia (Walder *et al.*, 1997). Subsequently, a number of other studies have confirmed this finding and implicated Nox2-NADPH oxidase-derived ROS in oedema formation, blood brain barrier dysfunction and excessive ROS production (Brait *et al.*, 2010; Jackman *et al.*, 2009a; Kahles *et al.*, 2007; Kunz *et al.*, 2006; Wang *et al.*, 2006). For example, following cerebral ischaemia Nox2-NADPH oxidase-derived H_2O_2 has been shown to cause cerebral endothelial cell contraction, which may contribute to blood brain barrier dysfunction and oedema formation (Kahles *et al.*, 2007). Therefore, Nox2-NADPH oxidase is a mediator of neurological damage and blood brain barrier dysfunction following cerebral ischaemia. However, it is currently unclear if Nox2-NADPH oxidase-derived ROS contribute to impaired NO· function in cerebral arteries following cerebral ischaemia-reperfusion.

More recently, it has become apparent that cerebral ischaemia induces a systemic inflammatory response. Leucocytes have been shown to enter the brain following cerebral ischaemia and cause tissue damage (Brait *et al.*, 2010; Gelderblom *et al.*, 2009; Hurn *et al.*, 2007; Yilmaz *et al.*, 2006). Furthermore, Nox2-NADPH oxidase has been suggested to be an important source of ROS in neurons, microglia and T-lymphocytes following cerebral ischaemia (Brait *et al.*, 2010; Kunz *et al.*, 2006; McCann *et al.*, 2008).

Influence of Gender on Cerebrovascular Function

It is well documented that gender influences the incidence and severity of several vascular diseases that affect the cerebral circulation, including hypertension and stroke (Appelros *et al.*, 2009; Prencipe *et al.*, 1997). For example, pre-menopausal women experience fewer strokes compared with men of similar age and post-menopausal women (Appelros *et al.*, 2009; Prencipe *et al.*, 1997). Currently, the precise mechanisms that contribute to these gender-dependent differences in the incidence of vascular diseases are not fully understood and are likely to be multifaceted. Interestingly, evidence suggests that gender-dependent differences in vascular ROS production may play an important role in the clinically observed differences in disease incidence. For example, clinical studies have reported that commonly

used markers of oxidative stress, including malondialdehyde and 8-isoprostaglandin $F_{2\alpha}$, are lower in healthy premenopausal women than age-matched men (Ide *et al.*, 2002; Powers *et al.*, 2002). Furthermore, experimental evidence shows that marked gender dependent differences exist in cerebral artery ROS production and vasoactive responses (Girouard *et al.*, 2008; Miller *et al.*, 2007b). Indeed, it has recently been demonstrated that NADPH–stimulated O_2^- production by cerebral arteries from male rats and mice is more than 2-fold greater compared with females under physiological conditions (Miller *et al.*, 2007a; Miller *et al.*, 2007b). Furthermore, this gender-dependent difference is associated with lower protein expression of the NADPH oxidase Nox1 and Nox4 catalytic subunits (Miller *et al.*, 2007b). Thus, it seems likely that lower expression of these isoforms of NADPH oxidase may contribute to the relatively lower level of O_2^- production in females. More recently, it has also been demonstrated that angiotensin II administration impairs NO·-dependent dilatation by cerebral arteries from male, but not female mice (Chrissobolis *et al.*, 2010; Girouard *et al.*, 2008), suggesting that cerebral arteries from females are protected from the deleterious effects of angiotensin II. As discussed, Nox2-NADPH oxidase is a critical mediator of the deleterious effects of angiotensin II on the cerebral circulation. As such, the aforementioned gender-dependent differences in response to angiotensin II may reflect differences in vascular ROS production by Nox2-NADPH oxidase. However, this is yet to be definitively tested. Thus, taken together, clinical and experimental data suggest that levels of ROS during normal physiological, and possibly pathological conditions, may be lower in females than males. Furthermore, this may be due to lower activity and/or expression of NADPH oxidases in females.

It is well established that gonadal sex hormones (i.e. oestrogens, progestins and androgens) can influence vascular function. In particular, numerous studies have investigated the effects of oestrogen on cerebral artery function (Capone *et al.*, 2009; Chrissobolis *et al.*, 2004b; Cipolla *et al.*, 2009a; Geary *et al.*, 1998; Girouard *et al.*, 2008; Miller *et al.*, 2007b). For example, recent work has demonstrated that NADPH-stimulated O_2^- production by cerebral arteries from female rats is elevated following ovariectomy (OVX), and this was restored to normal by 17β-oestradiol hormone replacement therapy (Miller *et al.*, 2007b), suggesting that oestrogen normally suppresses NADPH oxidase activity in the cerebral

circulation. Furthermore, a more recent study provided evidence to suggest that the vascular dysfunction induced by angiotensin II treatment is dependent on the oestrous cycle (Capone *et al.*, 2009). Specifically, when oestrogen levels are high, NO·-dependent dilatation is preserved and O_2^- production is suppressed, whereas when oestrogen levels are low, NO·-dependent dilatation is impaired and O_2^- production is augmented (Capone *et al.*, 2009). Taken together, experimental evidence indicates that the protective effect of the female gender on cerebral artery function may be due the ability of oestrogen to suppress NADPH oxidase-derived ROS production.

Aims of this Thesis

It is expected that the work presented in this thesis will provide understanding of the role(s) of Nox2-NADPH oxidase-derived ROS in cerebral arteries during physiological and pathological conditions. Specifically, the aims of this thesis were to:

1. Determine if gender influences cerebral artery ROS production and constrictor responses to angiotensin II, and the role of Nox2-NADPH oxidase (if any) in these responses (Chapter 3).

2. Elucidate the mechanisms by which angiotensin II elicits constriction of middle cerebral arteries (Chapter 4).

3. Investigate the effect of hypercholesterolaemia on cerebral artery ROS production and NO· function and the role of Nox2-NADPH oxidase in any such dysfunction (Chapter 5).

4. To determine if Nox2-NADPH oxidase contributes to augmented cerebral artery ROS production and impaired NO· function 24 h after transient cerebral ischaemia (Chapter 6).

CHAPTER 2

GENERAL METHODS

Animals

All procedures were approved by the Monash University School of Biomedical Sciences Animal Ethics Committee. In total 282 C57Bl6/J wild type mice (212 male and 72 female), 58 Nox2-deficient ($Nox2^{-/-}$; 45 male and 13 female), 65 apolipoprotein E-deficient mice ($ApoE^{-/-}$; male), 55 $Nox2^{-/-}/ApoE^{-/-}$ mice (male) and 7 p47phox-deficient ($p47phox^{-/-}$) mice (male) were used for the experiments detailed throughout this thesis. Unless otherwise stated, all mice were studied from 8-12 weeks of age.

C57Bl6/J Wild Type Mice

C57Bl6/J mice were obtained from Monash Animal Services (Monash University, Melbourne). Animals were housed in an approved animal holding facility with *ad libitum* access to standard rodent chow (4.8 % fat, 0.02 % cholesterol) and water. For experiments detailed in Chapter 5, some C57Bl6/J wild type mice were maintained on a high fat diet (21 % fat, 0.15 % cholesterol; Specialty Feeds, Australia) from five weeks of age for 7-14 weeks and housed at the Monash Mouseworks Facility.

Nox2-deficient Mice

$Nox2^{-/-}$ mice were purchased from Ozgene (Australia) and with C57Bl6/J mice. Genotypes were determined by Polymerase Chain Reaction (PCR) amplification of tail DNA and separate colonies of $Nox2^{-/-}$ mice and genetically related C57Bl6/J wild type mice were established. Mouse colonies were housed and bred at the Monash Mouseworks Facility with *ad libitum* access to standard rodent chow and water. For experiments detailed in Chapter 5, some $Nox2^{-/-}$ mice were maintained on a high fat diet from five weeks of age for 7-14 weeks.

Apolipoprotein E-deficient Mice

$ApoE^{-/-}$ mice were purchased from the Animal Resources Centre (Western Australia, Australia) and bred with C57Bl6/J mice. Genotypes were determined by PCR amplification of tail DNA and separate colonies of $ApoE^{-/-}$ mice and genetically related C57Bl6/J wild type mice were established. Mice were housed and bred at Monash Mouseworks Facility with *ad libitum* access to standard rodent chow and water until five weeks of age, after which mice were maintained of a high fat diet for 7-14 weeks.

Nox2-deficient/Apolipoprotein E-deficient Mice

$Nox2^{-/-}/ApoE^{-/-}$ mice were generated in our laboratory as described below. Female $Nox2^{-/-}/ApoE^{+/+}$ mice were bred with male $Nox2^{+/+}/ApoE^{-/-}$ mice to produce an F1 generation. The F1 generation was interbred generating an F2 generation of male and female wild type ($Nox2^{+/+}/ApoE^{+/+}$), $ApoE^{-/-}$ ($Nox2^{+/+}/ApoE^{-/-}$) and $Nox2^{-/-}/ApoE^{-/-}$ mice. Genotypes were determined by PCR amplification of tail DNA and a colony of $Nox2^{-/-}/ApoE^{-/-}$ mice was established with breeding pairs from the F2 generation. The $Nox2^{-/-}/ApoE^{-/-}$ mouse colony was genetically related to the C57Bl6/J wild type, $Nox2^{-/-}$ and $ApoE^{-/-}$ mouse colonies. Mice were housed and bred at Monash Mouseworks Facility with *ad libitum* access to standard rodent chow and water until five weeks of age, after which mice were maintained on a high fat diet for 7-14 weeks.

p47phox-deficient Mice

$p47phox^{-/-}$ mice were purchased from Taconic Labs (USA). Genotypes were determined by PCR amplification of tail DNA. Mouse colonies were housed and bred at the Monash Mouseworks Facility with *ad libitum* access to standard rodent chow and water.

Arteries Studied

Mice were euthanased by overdose of isoflurane inhalational anaesthetic (2-4 % in O_2; Baxter Healthcare, Australia) and then decapitated. Brains were rapidly removed and placed in ice cold Krebs-bicarbonate (for functional experiments; composition in mmol/L: NaCl 118, KCl 4.5, $MgSO_4$ 0.45, KH_2PO_4 1.03, $NaHCO_3$ 25, glucose 11.1, $CaCl_2$ 2.5) or Krebs-HEPES (for all other experiments; composition in mmol/L: NaCl 99.01, KCl 4.69, $CaCl_2$ 1.87, $MgSO_4$ 1.20, K_2HPO_4 1.03, $NaHCO_3$ 25.0, Na-HEPES 20.0, glucose 11.1, pH 7.4). With the aid of a dissecting microscope, the basilar artery was carefully cleared of excess connective tissue at its origin with the vertebral artery and gently dissected free of the cerebellum (Figure 1, arrow). Middle cerebral arteries (MCA) were cleared of any excess connective tissue and gently dissected free from the temporal lobe (Figure 1, arrow head).

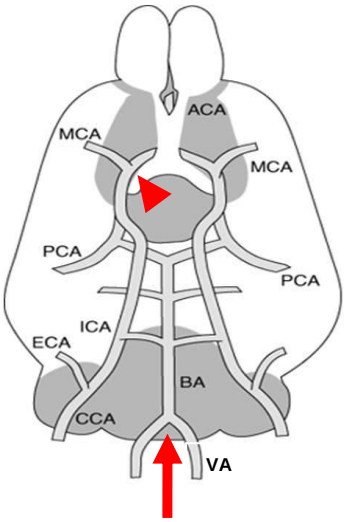


Figure 1: Diagram of arteries on the ventral surface of the mouse brain. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; VA, vertebral artery; ICA, internal carotid artery; ECA, external carotid artery; CCA, common carotid artery. Adapted from (O'Neill *et al.*, 2000).

Quantification of Superoxide Production by Cerebral Arteries

Lucigenin-enhanced Chemiluminescence

Lucigenin-enhanced chemiluminescence is a widely used technique for the measurement of superoxide (O_2^-) production by vascular tissue. Lucigenin is cell-permeable and is therefore able to detect O_2^- generated both intracellularly and extracellularly (Dikalov *et al.*, 2007). The detection of O_2^- by lucigenin is a multi step process. Lucigenin is reduced by two molecules of O_2^- forming the energy rich molecule dioxetane, which rapidly breaks down to 2*N*-methylacridone, releasing a photon in the process (Dikalov *et al.*, 2007). It has been suggested that O_2^- detected by lucigenin may be overestimated due to a phenomenon known as redox cycling, which is when lucigenin itself oxidises O_2, generating O_2^- (Liochev *et al.*, 1997). However, it has since been demonstrated that at lower concentrations of lucigenin (e.g. 5 µmol/L), the contribution of redox cycling to total O_2^- detected is negligible (Li *et al.*, 1998).

Stimulated O_2^- production by cerebral arteries (one MCA and half of a basilar artery) was measured by 5 µmol/L lucigenin-enhanced chemiluminescence. Cerebral arteries were excised as described above and then incubated with drug treatments (e.g. angiotensin II, indomethacin) in Krebs-HEPES solution for 30 min at 37 °C. Arteries were then transferred in semi-darkness to a Costar 96 well assay plate (Corning, USA) containing the same drug treatments as they were incubated in and 5 µmol/L of lucigenin dissolved in Krebs-HEPES. Photon counts were measured using a Topcount NXT plate reader (PerkinElmer; 25 °C, 3 s

per well for 20 cycles). Following the completion of the experiment, arteries were removed from the assay plate and dried overnight at 37 °C. Dry tissue weight was measured using a Mettler Toledo MT5 microbalance (Mettler Toledo). In all experiments, background counts were subtracted and O_2^- production (counts/mg dry tissue weight) normalised to dry tissue weight. Experimental protocols are described in detail in the methods section of appropriate chapters.

L-012-enhanced Chemiluminescence

L-012 is a luminol derivative that has been found to be a more sensitive chemiluminescence probe, compared with lucigenin, for the detection of O_2^- (Dikalov *et al.*, 2007). The greater sensitivity of L-012 facilitates the measurement of basal O_2^- production by vascular tissue, which is too low to be detected using lucigenin-enhance chemiluminescence. It has been suggested that L-012 may not be specific for O_2^- and it may also detect other reactive oxygen species, such as peroxynitrite ($ONOO^-$) (Daiber *et al.*, 2004). However, a recent study has concluded that L-012 specifically detects O_2^-, as treatment of mouse aortic segments with ebselen ($ONOO^-$ scavenger) had no effect on O_2^- production (Judkins *et al.*, 2009). Furthermore, L-012 appears to primarily detect extracellular O_2^-, as treatment of artery segments with native O_2^- dismutase (SOD; extracellular O_2^- scavenger) decreased the O_2^- detected to the same degree as treatment with tempol (an intracellular and extracellular O_2^- scavenger) (Judkins *et al.*, 2009).

Basal O_2^- production by cerebral arteries (one MCA and half a basilar artery) was measured by 100 µmol/L L-012-enhanced chemiluminescence. Cerebral arteries were excised as described above and transferred in semi-darkness to a Costar 96 well plate (Corning, USA) containing 100 µmol/L L-012 dissolved in Krebs-HEPES. Photon counts were read using a Hidex Chameleon V plate reader (Hidex Ltd., Finland; 25 °C, 3 seconds per well for 20 cycles). In some experiments, O_2^- production was measured in the presence of drug treatments (e.g. angiotensin II, N^{ω}-nitro-L-arginine methyl ester; L-NAME). Following the completion of the experiment, arteries were removed from the assay plate and dried overnight at 37 °C. Dry tissue weight was measured using a Mettler Toledo MT5 microbalance (Mettler Toledo). In all experiments, background counts were

subtracted and O_2^- production (counts/mg dry tissue weight) normalised to dry tissue weight. Experimental protocols are described in detail in the methods section of appropriate chapters.

Quantification of Hydrogen Peroxide Production by Cerebral Arteries

The Amplex Red fluorescence bioassay has been recently developed for the detection of hydrogen peroxide (H_2O_2) production by vascular tissues and cells. The assay involves the conversion of the nonfluorescent Amplex Red reagent reagent (N-acetyl-3,7-dihydroxyphenoxazine) to the highly fluorescent resorufin by horseradish peroxidise (HRP), which occurs only in the presence of H_2O_2 (Zhou *et al.*, 1997). Resorufin is a stable compound, and therefore the Amplex Red fluorescence bioassay is a measure of H_2O_2 production over the duration of the assay period. H_2O_2 is readily diffusible across cell membranes and reaches equilibrium between intracellular and extracellular compartments. Therefore, despite the Amplex Red reagent not being cell permeable, it is a reliable measure of both intracellular and extracellular H_2O_2 production by vascular tissue (Dikalov *et al.*, 2007).

The Amplex Red fluorescence bioassay was used to measure H_2O_2 production by cerebral arteries (one MCA and half a basilar artery). Cerebral arteries were excised as described above and transferred to a black 96-well plate (Nunc, Denmark) containing 15 μmol/L Amplex Red, 0.1 units/ml HRP and drug treatment (e.g. angiotensin II) dissolved in Krebs-HEPES. Blanks (Krebs-HEPES only) and H_2O_2 standards (H_2O_2 concentration in μmol/L: 0, 0.039, 0.078, 0.156, 0.312, 0.625), containing Amplex Red reagent and HRP, were run alongside cerebral artery samples. Fluorescence was measured in a fluorimeter (Flexstation, Molecular Devices; 37 °C, 30 cycles, 2 min between cycles) using an excitation filter of 530 nm and an emission filter of 590 nm running SOFTmax PRO software (Version 5.3; Molecular Devices, USA). Following the completion of the experiment, arteries were removed from the assay plate and dried overnight at 37 °C. Dry tissue weight was measured using a Mettler Toledo MT5 microbalance (Mettler Toledo). A H_2O_2 standard curve was constructed for each assay. Background fluorescence was subtracted, H_2O_2 accumulation (pmoles/min) was interpolated from the H_2O_2 standard

curve and subsequently normalised for dry tissue weight. Experimental protocols are described in detail in the methods section of specific chapters.

Preparation of Isolated Cannulated Middle Cerebral Artery Segments for Vascular Reactivity Studies

MCA were mounted between two microcannulae in a pressure myograph (Living Systems Instrumentation Inc., USA). Arteries were constantly superfused with warm (37 °C), carbogen-bubbled (95 % O_2, 5 % CO_2) Krebs-bicarbonate solution. Intraluminal pressure was gradually increased to 60 mmHg, to mimic pressure in the cerebral circulation *in vivo* (Faraci, 2003), and maintained at this level with a pressure servo unit without further intraluminal perfusion. Following equilibration, tissue viability was assessed by exposure to a high potassium physiological salt solution (KPSS) containing 122.7 mmol/L KCl (equimolar replacement of NaCl with KCl). Arteries were viewed through a Nikon Eclipse TS100 inverted microscope fitted with a 10× objective lens and Sony XC-ST30CE CCD video camera module (Sony, Japan). Vessel diameter was measured by a V-94 Video Dimension Analyser (Living Systems Instrumentation Inc., USA) and connected to a DATAQ Instruments DI-720 data acquisition device and a PC running Windaq Pro software (Version 3.07; DATAQ Instruments, USA). Experimental protocols are described in detail in the methods section of specific chapters.

Western Blotting

Tissue Homogenisation

Cerebral arteries were excised as described above and snap frozen in liquid nitrogen. Frozen cerebral arteries were crushed over liquid nitrogen before the addition of Laemmli buffer (7.5 % glycerine, 3.75 % β-mercaptoethanol, 2.25 % sodium dodecyl sulphate, 0.75 mol/L Tris-HCl pH 8.0, 0.075 mg/ml bromophenol blue). Samples were then sonnicated on ice, heated at 37 °C for 10 min followed by centrifugation (15,000 rpm for 10 min at 4 °C). Five µl of sample was taken for determination of protein concentration and the remainder was stored at -80 °C until use. Protein concentration was determined using the *RC DC* protein assay (BioRad, USA) as per the manufacturer's directions.

Protein Electrophoresis

Equal amounts of protein and dual colour molecular weight marker (BioRad, USA) were loaded onto 1 mm thick polyacrylamide gels (4 % acrylamide for upper gel and 7.5 % acrylamide for lower gel) and mounted in a BioRad mini PROTEAN® 3 cells. Voltage was set at 60 V for migration of protein through the upper gel and raised to 95 V for lower gel.

Transfer of Proteins to Membrane

Following electrophoresis, gels were carefully removed from glass plates and placed in transfer buffer 2 (0.025 mol/L Tris-base, 20 % methanol**,** pH 10.4) on a shaker for 10 min. Immobilon-P Polyvinylidene Fluoride (PVDF) transfer membranes (Milipore, USA) were placed in methanol for 10 sec, rinsed with distilled H_2O for 5 min followed by transfer buffer 2 for 10 min. Two pieces of Whatmans blotting paper (Whatmans Inc., United Kingdom) was soaked in transfer buffer 1 (0.3 mol/L Tris-base, 20 % methanol, pH 10.4), one piece in transfer buffer 2 and two pieces in transfer buffer 3 (0.038 mol/L Tris-base, 0.01 mol/L β-alanine, 20 % methanol, pH 9.4). Blotting paper, PVDF membrane and gel were arranged in a TE 77 Semi Dry Transfer Unit (Amersham Biosciences, USA) as shown in Figure 2 and transferred by the semi dry transfer method (95 mA per gel for 70 min). Successful protein transfer was confirmed by Ponceau S staining before washing with Tris Buffered Saline containing 0.1 % Tween 20 (TBS-T; 20 mmol/L Tris, 15 mmol/L NaCl, 0.1 % Tween 20).

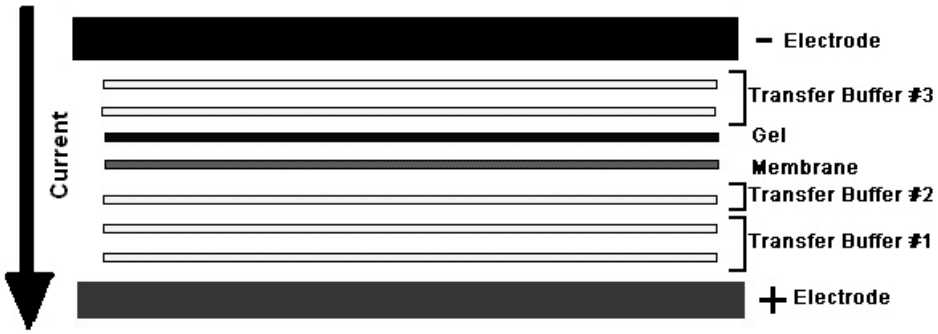


Figure 2: Layout of blotting paper sheets, gel and membrane for semi-dry transfer of proteins to PVDF membrane.

Blocking and Antibody Incubation

Membranes were incubated in 5 % skim milk dissolved in TBS-T for 1 hour at room temperature to reduce non-specific binding of antibodies. Membranes were incubated overnight (4 °C) with the appropriate primary antibody (as described in the methods section of each specific results chapter) in 5 % skim milk solution with constant gentle agitation. The following day, membranes were washed 3×15 min with TBS-T. Following washes, membranes were incubated with a HRP–conjugated secondary antibody made up in 5 % skim milk solution for 1 hour at room temperature with constant gentle agitation followed by 3×15 min washes with TBS-T.

Enhanced Chemiluminescence Visualisation

The membrane was saturated in Immobilon Western enhanced chemiluminescence HRP substrate (Millipore, USA). Membrane was wrapped in a plastic pocket and placed in a hypercassette. In a dark room, photographic paper (Fujifilm SuperRx; Fujifilm, Japan) was placed over the membrane and exposed for between 30 s and 30 min, depending on the antibody used. The film was then developed with an AGFA CP1000 processor (AGFA, Germany) and the intensity of the immunoraective band was quantified using a ChemiDoc XRS molecular imager (BioRad, USA).

Stripping of Membrane for Detection of β-actin

In all experiments, the intensity of the protein of interest was normalised to the intensity of its corresponding band for β-actin. After visualisation of bands, membranes were

stripped by washing with 0.5 mol/L NaOH. Membranes were then washed 3×15 min with TBS-T. To confirm successful removal of antibodies, enhanced chemiluminescence visualisation was performed as described above. Membranes were then washed for 10 min with TBS-T, blocked with 5 % skim milk, incubated with anti- β-actin primary antibody and appropriate secondary antibody and visualised as described above. The anti-β-actin antibody used was raised in a different species to the original protein of interest, thus preventing the binding of the secondary antibody to any residual original primary antibody.

Preparation of Cerebral Arteries for Histological Analysis

The temporal lobe with the MCA attached and the cerebellum with the basilar artery attached were embedded in OCT Tissue-Tek (Sakura Finetek, USA) and frozen in isopentane over liquid nitrogen. MCA and basilar arteries were then sectioned (10 µm) using a Leica CM-1850 cryostat (Leica MicroSystems, Germany) and thaw-mounted onto slides coated with 0.1 % poly-L-lysine. Sections were allowed to air dry for 30 min and stored at -80 °C until use. Specific histological protocols are described in the methods section of relevant results chapters.

Immunofluorescence

MCAs were prepared for histological analysis as described above. Sections were fixed in ice-cold acetone (15 min) and washed in 0.01 mol/L phosphate buffered saline (PBS; 3x10 min; composition in mmol/L: Na_2HPO_4 5, NaH_2PO_4 5, NaCl 154, pH 7.4). Sections were then incubated with the appropriate primary antibody (as described in the methods section of each specific results chapter) overnight at 4 °C. The following day, the tissues were washed in PBS (3x10 min) and incubated with a fluorescently labelled secondary antibody (as described in the methods section of each specific results chapter) for 3-4 h at 4 °C. The tissues were then washed in PBS (3x10 min) and mounted in buffered glycerol (0.5 mol/L Na_2CO_3 added dropwise to 0.5 mol/L $NaHCO_3$ to pH 8.6, combined 1:1 with glycerol). Imaging was performed using a Leica TCS upright confocal microscope equipped with a 40× oil immersion lens and running Leica Confocal Software (Leica MicroSystems, Germany).

Drugs and Chemicals

Amplex Red fluorescence bioassay kit was purchased from Molecular Probes (Invitrogen; USA), angiotensin II from Auspep (Australia), L-012 from Wako Pure Chemicals (Japan) and all other drugs were from Sigma Aldrich or Merck.

Angiotensin II was dissolved in 0.05 mol/L acetic acid. L-012 was prepared in 100 mmol/L in dimethyl sulfoxide (DMSO) and diluted in Krebs-HEPES solution, such that the final concentration of DMSO was ≤0.1 %. All other drugs were dissolved and/or diluted in either Krebs-HEPES (lucigenin/L012-enhanced chemiluminescence and Amplex Red fluorescence bioassay experiments) or Krebs-bicarbonate (functional experiments).

Data Analysis

All results are presented as mean ± SEM. Statistical comparisons were performed using one-way ANOVA with a Bonferroni multiple comparison post-hoc test, a two-way ANOVA, Mann-Whitney test or paired/unpaired *t* test, as indicated in the appropriate results chapters. $P<0.05$ was considered statistically significant.

CHAPTER 3

GENDER INFLUENCES CEREBRAL VASCULAR RESPONSES TO ANGIOTENSIN II THROUGH NOX2-DERIVED REACTIVE OXYGEN SPECIES

Introduction

Hypertension has devastating effects on the cerebral circulation and is a major risk factor for ischaemic (including lacunar strokes) and haemorrhagic stroke (Jackson *et al.*, 2010; Lewington *et al.*, 2002; O'Donnell *et al.*, 2010) as well as Alzheimer's disease (Kivipelto *et al.*, 2001). Angiotensin II is believed to be a critical mediator of the deleterious effects of hypertension on the cerebral circulation. For example, it has been reported to promote cerebrovascular remodelling (Baumbach *et al.*, 2003) and inflammation (Ando *et al.*, 2004), as well as impairing functional hyperaemia (Capone *et al.*, 2010a; Girouard *et al.*, 2006; Kazama *et al.*, 2004). NADPH oxidases are a major source of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in the cerebral circulation (Miller *et al.*, 2005; Miller *et al.*, 2009). These enzymes generate ROS by transferring electrons to molecular oxygen via a "Nox" catalytic subunit. Nox1-, Nox2- and/or Nox4-containing isoforms of NADPH oxidase are thought to be important for the generation of ROS within the cerebral circulation (Miller *et al.*, 2006a). Importantly, evidence suggests that many of the deleterious effects of angiotensin II on the cerebral circulation are mediated through the excessive generation of ROS by Nox2-NADPH oxidase (Girouard *et al.*, 2006; Kazama *et al.*, 2004).

It is well documented that the incidence of cerebrovascular diseases such as stroke is lower in premenopausal women than men of similar age (Appelros *et al.*, 2009; Prencipe *et al.*, 1997). The precise mechanisms responsible for this difference are not fully understood and are likely to be multifaceted. However, recent findings suggest that gender-dependent differences in vascular ROS production may play an important role (Miller *et al.*, 2007a). Indeed, both the activity and expression of NADPH oxidase has been reported to be lower in the cerebral circulation of female versus male rats in an oestrogen-dependent manner (Miller *et al.*, 2007b). Moreover, studies have reported a marked gender-dependent difference in vasoconstrictor responses to angiotensin II (Faraci *et al.*, 2005). However, the role of Nox2-NADPH oxidase-derived ROS in such gender differences has not been evaluated. Therefore, we first tested whether gender influences the levels of ROS generated in cerebral arteries in response to angiotensin II, and then whether constrictor responses to angiotensin II were similarly gender-

dependent. Secondly, using Nox2 deficient ($Nox2^{-/-}$) mice we evaluated the involvement of Nox2-NADPH oxidase in these cerebrovascular responses to angiotensin II.

Materials and Methods

All procedures were approved by the institutional animal ethics committee. In total, 75 male C57Bl6/J wild type (24.5 ± 0.2 g), 72 female wild type (19.5 ± 0.2 g), 16 male $Nox2^{-/-}$ (23.2± 0.5 g) and 13 female $Nox2^{-/-}$ (20.1± 0.7 g) mice were studied. For L-012 experiments, male and female C57Bl6/J wild type mice were from the same parental lineage as $Nox2^{-/-}$ mice. All mice were bred at Monash University and housed in an approved animal holding facility. Mice were 8-12 weeks of age and were typically studied in pairs (i.e. male and female) that were age-matched on the day of each experiment.

Quantification of O_2^- Production by Cerebral Arteries

Lucigenin-enhanced Chemiluminescence

Angiotensin II (0.1 µmol/L) -stimulated O_2^- production by cerebral arteries (pooled basilar and MCA) from male and female wild type mice was measured by 5 µmol/L lucigenin-enhanced chemiluminescence as described in Chapter 2. Cerebral arteries were excised and incubated with NADPH (substrate for NADPH oxidase; 100 µmol/L) + diethyldithiocarbamic acid (superoxide dismutase [SOD] 1/3 inhibitor, DETCA; 3 mmol/L) or NADPH + DETCA + angiotensin II (0.1 µmol/L) for 30 min at 37 °C. In some experiments, arteries were treated with the cyclooxygenase (COX) inhibitor, indomethacin (10 µmol/L) or the flavin antagonist/NADPH oxidase inhibitor, diphenyleneiodonium (DPI; 5 µmol/L). Counts from control arteries (NADPH and DETCA) were subtracted from counts from arteries in the presence of NADPH + DETCA + angiotensin II from the same animal, thereby giving O_2^- levels generated in response to angiotensin II. In experiments investigating the effects of indomethacin or DPI on angiotensin II-stimulated O_2^- production, counts from indomethacin or DPI treated arteries was normalised as a percentage of counts from control arteries. In all experiments, O_2^- production was normalised to dry tissue weight.

L-012-enhanced Chemiluminescence

Basal and angiotensin II (0.1 µmol/L) -stimulated O_2^- production by cerebral arteries from male and female wild type and $Nox2^{-/-}$ mice was measured by 100 µmol/L L-012-enhanced chemiluminescence as described in Chapter 2. Basal counts were subtracted

from counts in the presence of angiotensin II from the same animal, and O_2^- production was normalised to dry tissue weight.

Quantification of H_2O_2 production by Cerebral Arteries

The Amplex Red fluorescence bioassay was used to measure angiotensin II (0.1 µmol/L) -stimulated H_2O_2 production by cerebral arteries from male and female wild type mice as described in Chapter 2. Basal fluorescence was subtracted from fluorescence in the presence of angiotensin II, and H_2O_2 accumulation (pmoles/min) was normalised to dry tissue weight.

Vascular Reactivity Studies

Middle cerebral arteries (MCA) from male and female wild type, and $Nox2^{-/-}$ mice were mounted between two microcannulae in a pressure myograph (Living Systems Instrumentation Inc.) as described in Chapter 2. Following exposure to high potassium physiological salt solution (KPSS; 122.7 mmol/L K^+) and a washout period, cumulative concentrations of angiotensin II (0.1 nmol/L – 1 µmol/L, 5 min between additions) were added extraluminally to the MCA. Intraluminal diameter was tracked by a video dimension analyser and constrictor responses expressed as percent change in intraluminal diameter.

Western Blotting

Protein expression of Nox2 (antibody concentration 1:1000), SOD isoforms (SOD1, 1:4000; SOD2, 1:1000; SOD3, 1:1000) angiotensin receptor type 1 and 2 (AT_1R, 1:1000; AT_2R, 1:3000) and endothelial nitric oxide synthase (eNOS; 1:1000) was measured in cerebral arteries using Western blotting as described in Chapter 2. In all experiments, relative protein intensity was normalised to the corresponding band for β-actin (1:2000) as described in Chapter 2.

Localisation of Nox2

MCA from male and female wild type mice were mounted for histological analysis as described in Chapter 2. Sections were then fixed in ice cold acetone (15 min) and washed in 0.01 mol/L phosphate buffered saline (PBS; 3x10 min). Sections were then incubated

with an anti-Nox2 mouse monoclonal antibody (antibody concentration 1:1000) overnight at 4°C. The following day, the tissues were washed in 0.01 mol/L PBS (3x10 min) and incubated in a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (antibody concentration 1:500) for 3-4 h at 4°C. The tissues were then washed in 0.01 mol/L PBS (3x10 min) and mounted in buffered glycerol (0.5 mol/L Na_2CO_3 added dropwise to 0.5 mol/L $NaHCO_3$ to pH 8.6, combined 1:1 with glycerol). Tissue mounted slides were viewed and photographed on a Leica confocal scanning laser system.

Antibodies & Drugs

Anti-Nox2 and anti-eNOS mouse monoclonal antibodies were purchased from BD Biosciences (U.S.A). Anti-AT_1 and anti-AT_2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (U.S.A.), anti-SOD1 rabbit polyclonal antibodies from Stressgen (Canada), and anti-SOD2 anti-SOD3 rabbit polyclonal antibodies from Upstate Cell Signalling Solutions (U.S.A.). Anti-β-actin mouse/rabbit polyclonal antibodies were purchased from Cell Signaling Technology (U.S.A.) and FITC conjugated goat anti-mouse antibody from Zymed Laboratories (U.S.A.).

Angiotensin II was purchased from Auspep, the Amplex Red fluorescence bioassay kit from Molecular Probes (Invitrogen), L-012 from Wako Pure Chemicals (Japan) and all other drugs from Sigma. Angiotensin II was dissolved in 0.05 mol/L acetic acid and then diluted in either Krebs-HEPES (lucigenin/L-012 experiments) or Krebs bicarbonate (myograph experiments). DPI was prepared at 10 mmol/L in dimethyl sulfoxide (DMSO), and diluted in Krebs-HEPES solution. L-012 was prepared at 100 mmol/L in DMSO and subsequently diluted in Krebs-HEPES. All other drugs were dissolved and diluted in either Krebs-HEPES (lucigenin/L-012 experiments) or Krebs-bicarbonate (myograph experiments). For experiments using DPI or L-012, the final concentration of DMSO was ≤0.1 %.

Data Analysis

All results are presented as mean ± SEM. Statistical comparisons were performed using either two-way ANOVA, one-way ANOVA with a Bonferroni multiple comparison post-hoc

test or using paired or unpaired *t* test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Influence of Gender on Reactive Oxygen Species Production by Cerebral Arteries

Using 100 µmol/L L-012-enhanced chemiluminescence, basal O_2^- production by cerebral arteries was similar in male and female wild type mice (Figure 1A). Using 5 µmol/L lucigenin-enhanced chemiluminescence, angiotensin II increased O_2^- production by cerebral arteries from both genders (male, control 112.1 ± 36.2 vs. angiotensin II 172.1 ± 36.2; female, control 104.7 ± 15.4 vs. angiotensin II 124.7 ± 15.4, 10^3 counts/mg of dry tissue weight, n=9 for both genders). However, O_2^- production by cerebral arteries from female wild type mice was approximately 70 % lower than levels generated by arteries from male wild type mice (P<0.05, Figure 1B). The COX inhibitor, indomethacin had no significant effect on angiotensin II-stimulated O_2^- production by cerebral arteries from male mice (Figure 3A. control 115.6 ± 18.9 vs. indomethacin 175.4 ± 44.7, 10^3 counts/mg of dry tissue weight, n=6 for both groups). By contrast, the flavin antagonist/NADPH oxidase inhibitor, DPI virtually abolished angiotensin II-stimulated O_2^- production by cerebral arteries from male and female wild type mice (P<0.05, Figure 3B; male, control, 148.4 ± 36.4 vs. DPI, 7.6 ± 2.1; female, control, 125.4 ± 7.1 vs. DPI, 9.2 ± 6.7, 10^3 counts/mg of dry tissue weight, n=4 for male, n=3 for female). Consistent with the O_2^- data, angiotensin II-stimulated H_2O_2 production by cerebral arteries from female mice was approximately 85 % lower than levels generated by arteries from male mice (P<0.05, Figure 2).

Influence of Gender on Cerebral Artery Constrictor Responses to Angiotensin II

Baseline diameters of MCA were not significantly different between genders (Table 1). Angiotensin II (0.1 nmol/L – 1 µmol/L) elicited concentration-dependent constriction of MCA from both male and female wild type mice, however constriction was significantly smaller in MCA from female mice (P<0.05, Figure 4A). For example, at 1 µmol/L, angiotensin II constricted MCA from males by approximately 28 % compared with approximately 14 % in females. In contrast, constrictor responses to KPSS (also measured from baseline) were similar between genders (Figure 4B).

Role of Nox2-NADPH Oxidase in the Gender-dependent Differences in Response to Angiotensin II

Using L-012-enhanced chemiluminescence, angiotensin II-stimulated O_2^- production by cerebral arteries from female wild type mice was approximately 70 % lower than levels generated by arteries from male wild type mice (P<0.05, Figure 5). Interestingly, angiotensin II-stimulated O_2^- production by cerebral arteries from male Nox2$^{-/-}$ mice was found to be approximately 60 % lower compared with levels generated by arteries from male wild type mice (P<0.05, Figure 5). Indeed, levels in male Nox2$^{-/-}$ mice were comparable to levels generated by cerebral arteries from female wild type mice (P>0.05). By contrast, there was no difference between female Nox2$^{-/-}$ and female wild type mice with respect to cerebral artery O_2^- production (Figure 5).

Angiotensin II-induced constriction of MCA from male Nox2$^{-/-}$ mice was significantly smaller than responses of male wild type vessels (P<0.05, Figure 6A). For example, at 1 µmol/L angiotensin II constricted MCA from male Nox2$^{-/-}$ approximately 12 % compared with approximately 28 % in male wild type mice. Furthermore, constrictor responses of male Nox2$^{-/-}$ vessels were comparable to responses of MCA from female wild type mice (P>0.05, Figure 6A). In contrast, in females, responses to angiotensin II did not differ between Nox2$^{-/-}$ and wild type mice (Figure 6A). Constrictor responses to KPSS (also measured from baseline) were similar between all four groups (Figure 6B). Baseline diameters of MCA were similar between male and female Nox2$^{-/-}$. Furthermore, baseline diameters were not significantly different between genotypes (Table 1).

	Male Wild Type	**Male Nox2$^{-/-}$**	**Female Wild Type**	**Female Nox2$^{-/-}$**
Diameter (µm)	114 ± 5 (6)	122 ± 4 (6)	104 ± 8 (5)	120 ± 4 (6)

Table 1. Intraluminal diameters of isolated middle cerebral arteries from male and female wild type and Nox2$^{-/-}$ mice (n numbers given in brackets).

Nox2 Expression and Localization in Cerebral Arteries

Using Western blotting, Nox2 protein expression levels in cerebral arteries was found to be similar between genders (Figure 7). Using immunofluorescence, Nox2 immunoreactivity was predominantly observed in the endothelium and to a lesser extent

in the adventitia of arterial sections from both male and female mice (Figure 8). No specific Nox2 immunoreactivity was located in the vascular smooth muscle. Nox2 immunofluorescence intensity and distribution appeared similar in MCA from both genders.

SOD, AT Receptor and eNOS Expression in Cerebral Arteries

Using Western blotting, protein expression levels of SOD isoforms (SOD1, Figure 9A; SOD2, Figure 9B; SOD3, Figure 9C), AT_1 and AT_2 receptors (Figure 10A and 10B) and eNOS (Figure 10C) in cerebral arteries was similar between genders.

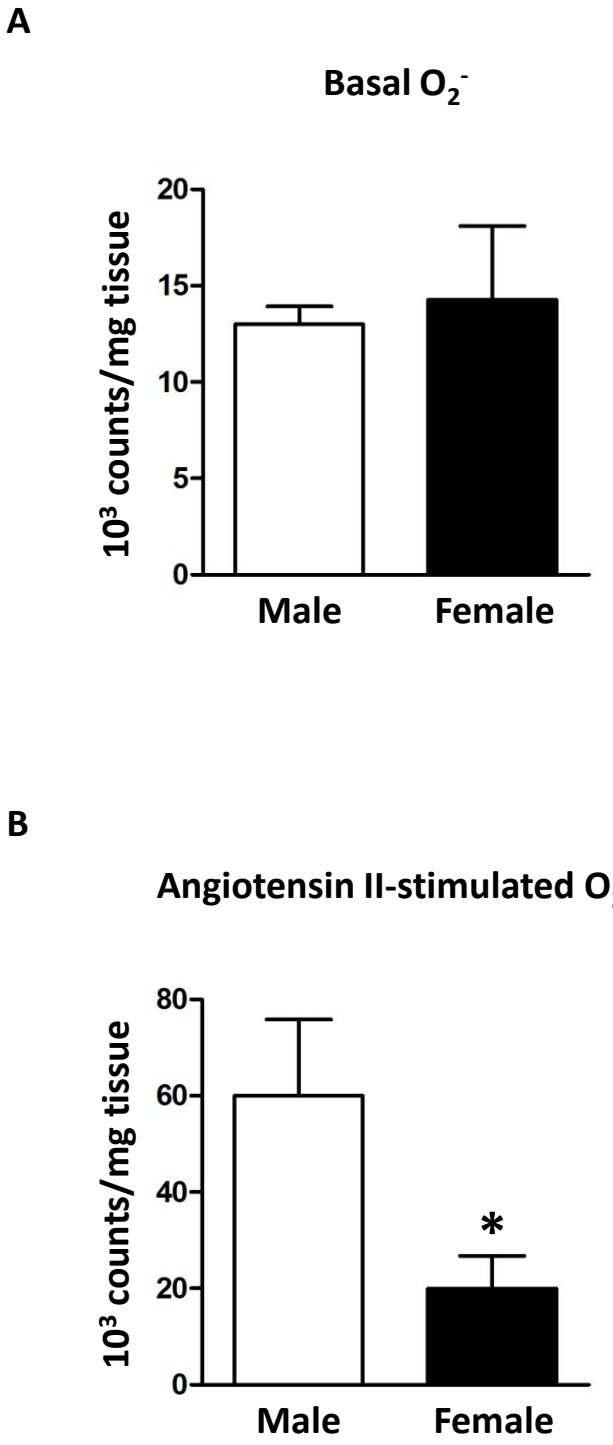


Figure 1. Basal O_2^- production by cerebral arteries from male and female wild type mice as measured by 100 µmol/L L-012-enhanced chemiluminescence **(A)**. Angiotensin II (0.1 µmol/L)-stimulated O_2^- production as measured by 5 µmol/L lucigenin-enhanced chemiluminescence **(B)**. All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (**A**, n=7 for both genders; **B**, n=9 for both genders). ***P*<0.05 vs. male (unpaired *t* test).

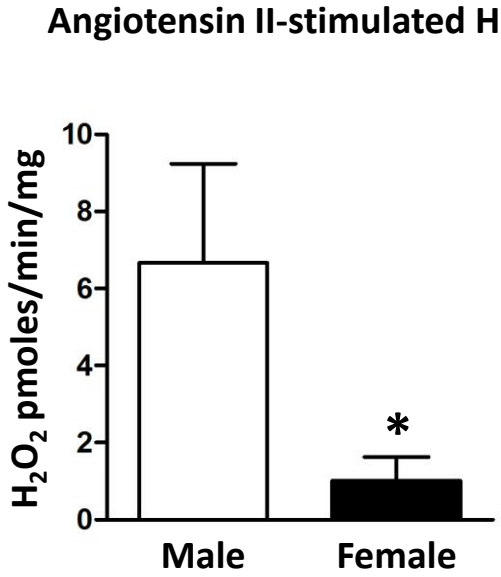


Figure 2. Angiotensin II (0.1 µmol/L)-stimulated H_2O_2 production by cerebral arteries from male and female wild type mice as measured by the Amplex Red fluorescence bioassay. The data represents counts in angiotensin II treated arteries minus basal counts in arteries from the same animal. Results are expressed in pmoles of H_2O_2 generated per min per mg of tissue and given as mean ± SEM (n=9 for both genders). ***P*<0.05 vs. male (unpaired *t* test).

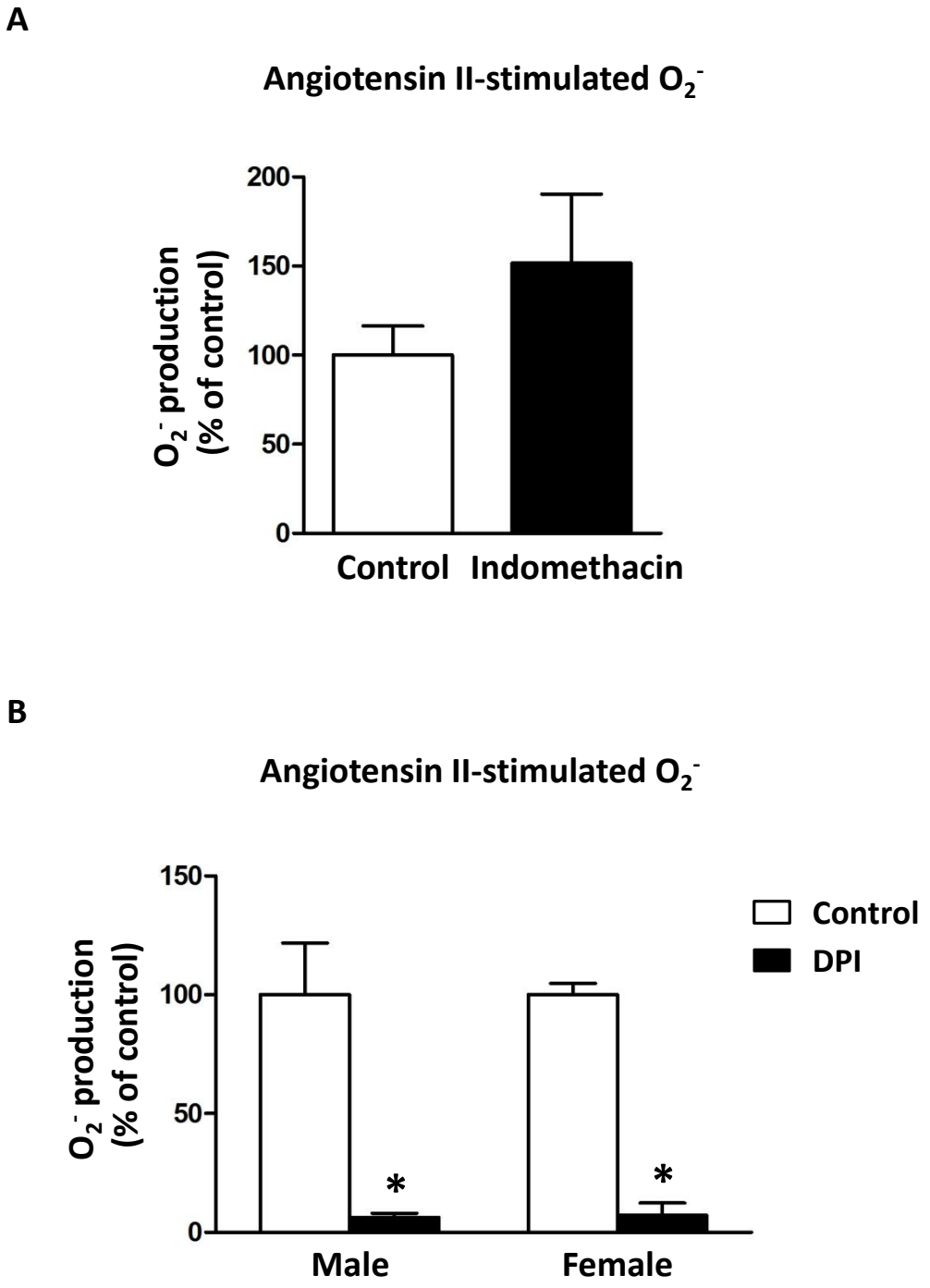
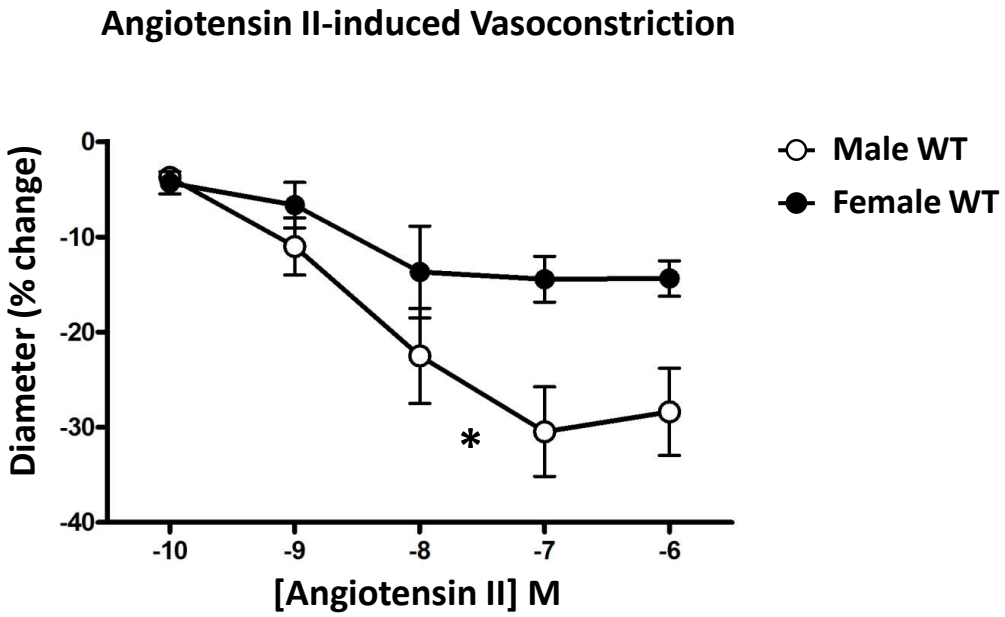


Figure 3. The effect of cyclooxygenase inhibitor indomethacin (10 µmol/L) on angiotensin II (0.1 µmol/L) -stimulated O_2^- production by cerebral arteries from male wild type mice as measured by 5 µmol/L lucigenin-enhanced chemiluminescence **(A)**. The effect of diphenyleneiodonium (DPI; 5 µmol/L) on angiotensin II (0.1 µmol/L)-stimulated O_2^- production by cerebral arteries from male and female wild type mice as measured by 5 µmol/L lucigenin-enhanced chemiluminescence **(B)**. All results given as mean ± SEM (**A** n=6 for both groups; **B** n=4 for male and n=3 for female). ***P*<0.05 vs. control (paired *t* test).

A



B

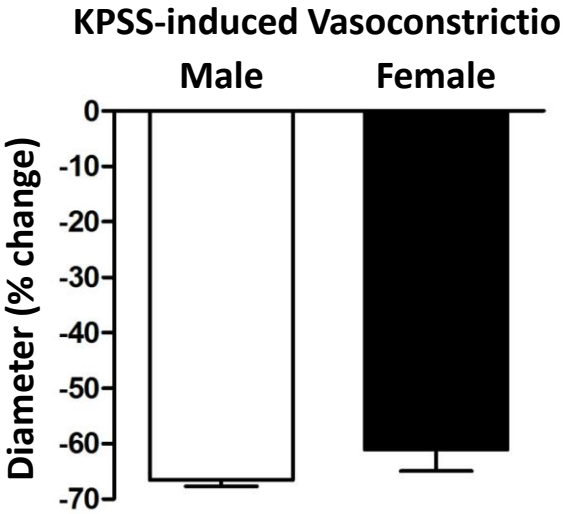


Figure 4. Cumulative concentration-response curves showing constrictor responses to angiotensin II (0.1 nmol/L – 1 µmol/L) of isolated middle cerebral arteries (MCA) from male and female wild type (WT) mice **(A)**. Constrictor responses of isolated MCA from male and female wild type mice to high potassium physiological salt solution (KPSS; 122.7 mmol/L) **(B)**. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (**A** and **B**, n=6 for male and n=5 for female). **P*<0.05 vs. concentration response curves for angiotensin II in MCA from female wild type mice (two-way ANOVA with a Bonferroni multiple comparison post-hoc test).

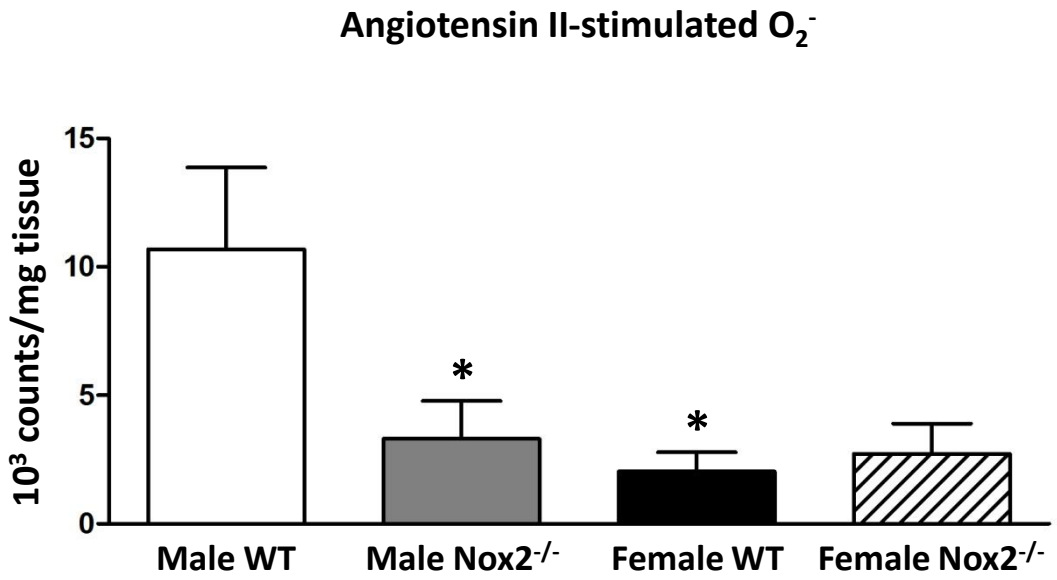


Figure 5. Angiotensin II (0.1 µmol/L)-stimulated O_2^- production by cerebral arteries from male and female wild type (WT) and Nox2-deficient ($Nox2^{-/-}$) mice as measured by 100 µmol/L L-012-enhanced chemiluminescence. The data represents counts in angiotensin II treated arteries minus basal counts in arteries from the same animal. All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (n=9 for male WT, n=10 for male $Nox2^{-/-}$, n=8 for female WT and n=7 for female $Nox2^{-/-}$). *****P<0.05 vs. male WT (one-way ANOVA with a Bonferroni's multiple comparison post-hoc test).

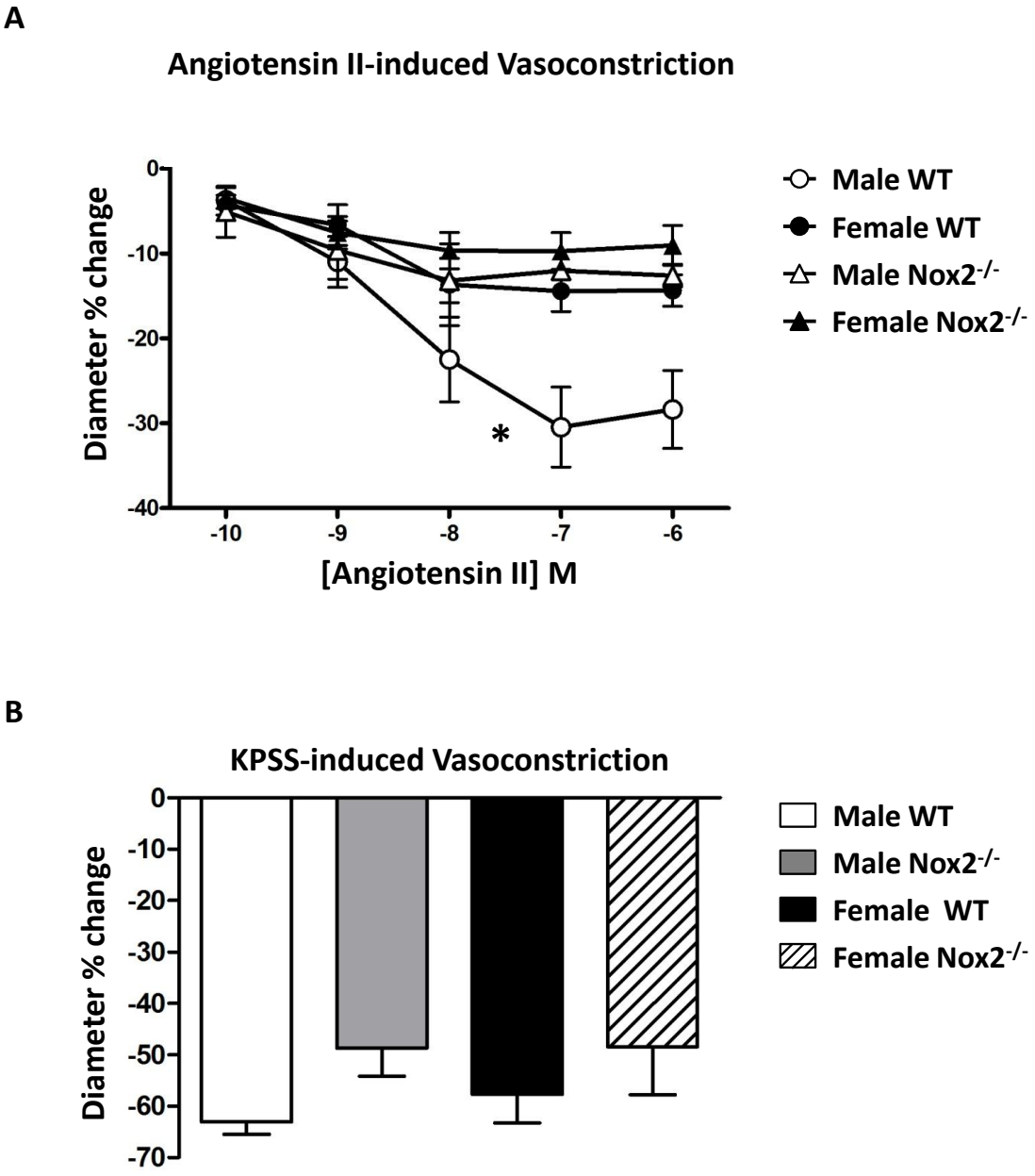


Figure 6. Cumulative concentration-response curves showing constrictor responses to angiotensin II (0.1 nmol/L – 1 µmol/L) of isolated middle cerebral arteries (MCA) from male and female wild type (WT) and Nox2-deficient ($Nox2^{-/-}$) mice **(A)**. Constrictor responses to high potassium physiological salt solution (KPSS; 122.7 mmol/L) **(B)**. Results are expressed as % change in intraluminal diameter, and given as mean ± SEM (**A** and **B**, n=6 for male WT, and male and female $Nox2^{-/-}$, n=5 for female WT). *P<0.05 vs. concentration response curves for angiotensin II in MCA from female WT and male and female $Nox2^{-/-}$ mice (two-way ANOVA with a Bonferroni multiple comparison post-hoc test). *Male and Female WT data are reproduced from Figure 4A and 4B for comparative purposes.*

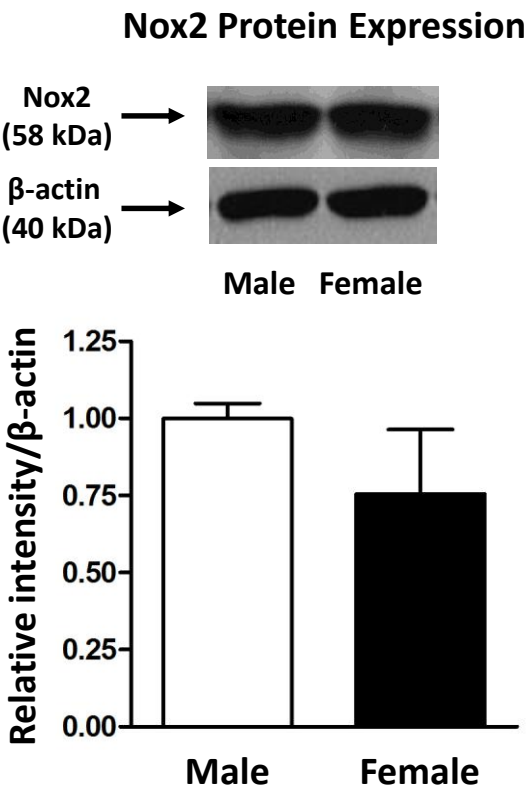


Figure 7. Representative western blot showing protein expression of the NADPH oxidase catalytic subunit Nox2 in cerebral arteries from male and female wild type mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM (n=6 for both genders).

Nox2 Protein Localisation

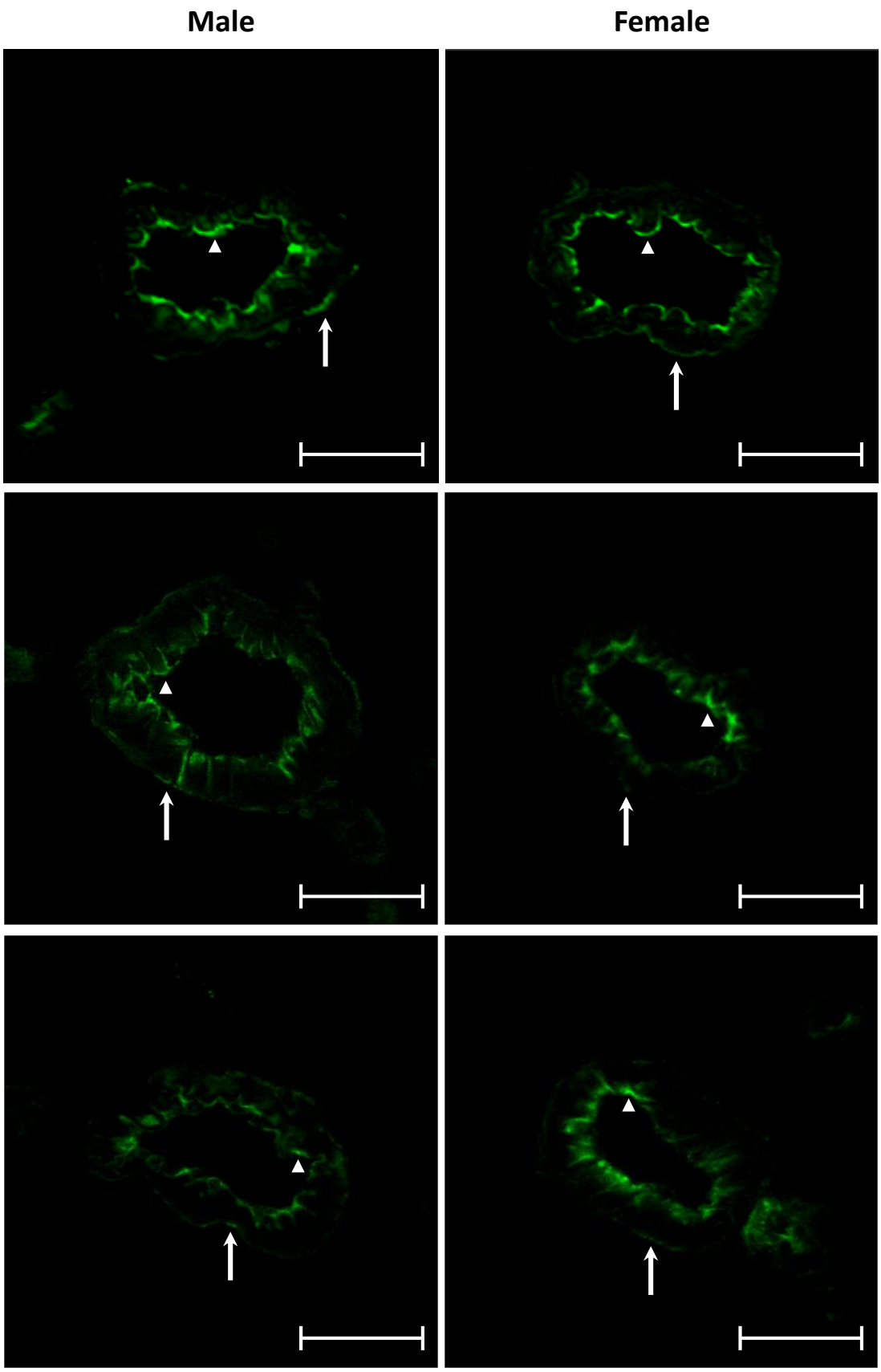


Figure 8. Photomicrographs showing sections of the middle cerebral artery from male and female wild type mice following processing for Nox2 immunofluorescence. In arterial sections from both males and females, Nox2-staining was observed in the endothelium (arrowheads) and adventitia (arrows). Magnification, 400×, scale bar=40 μm. n=3 for both genders.

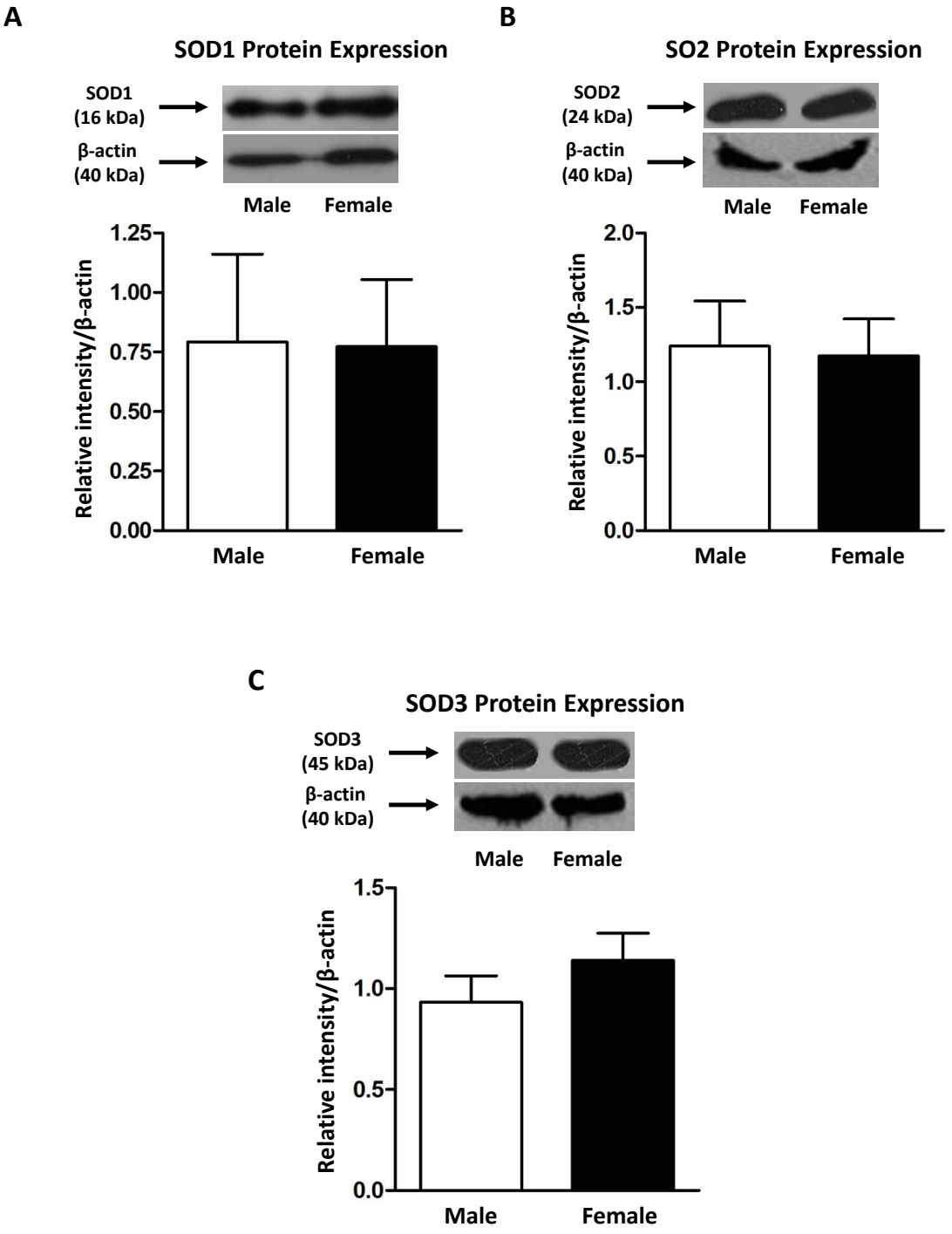


Figure 9. Representative western blots showing protein expression of superoxide dismutase (SOD) isoforms SOD1 **(A)**, SOD2 **(B)** and SOD3 **(C)** in cerebral arteries from male and female wild type mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM (**A**, n=7 for both genders; **B**, n=4 for male and n=5 for female; **C**, n=4 for male and n=5 for female).

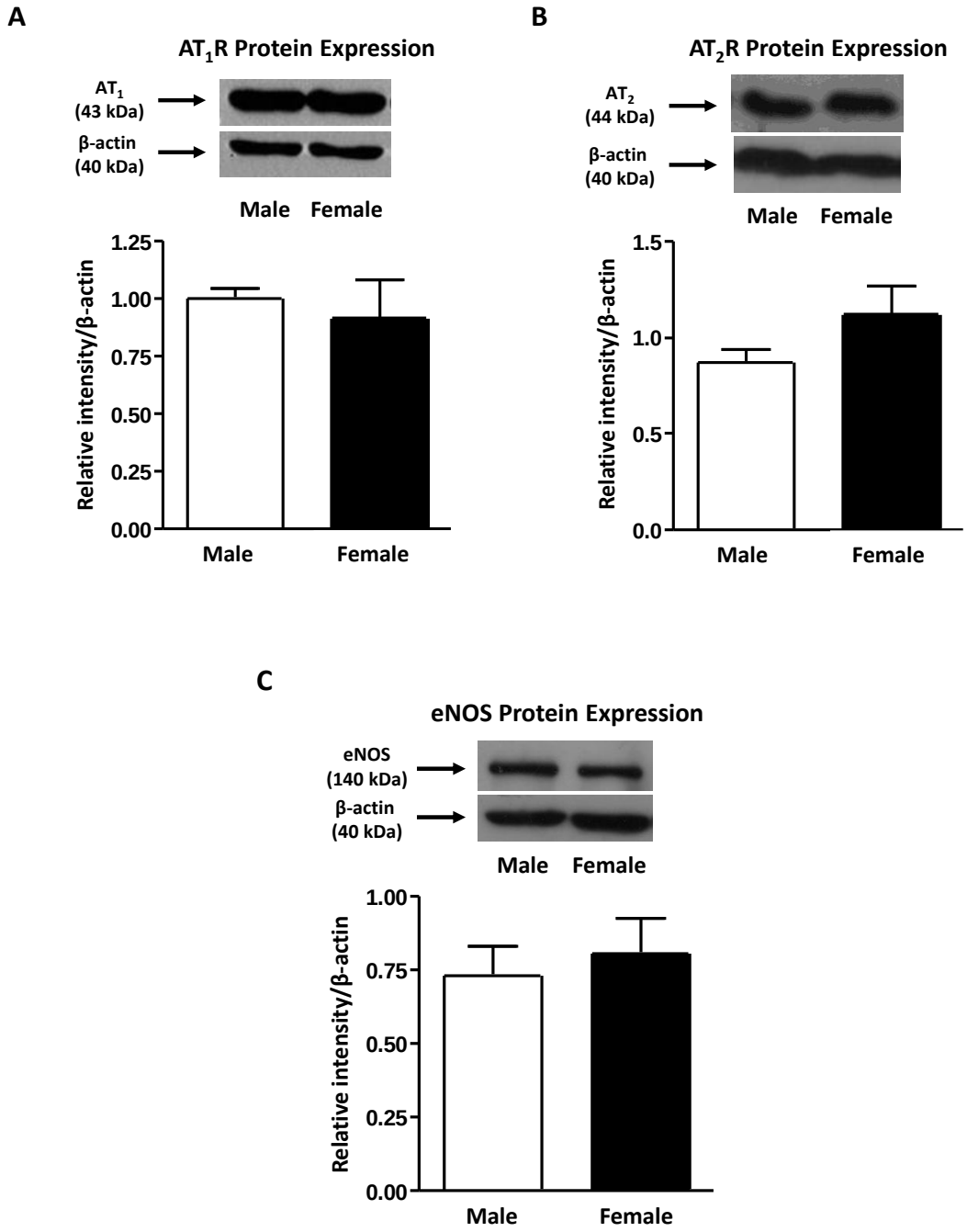


Figure 10. Representative western blots showing protein expression of the angiotensin receptor type 1 (AT_1R; **A**), angiotensin receptor type 2 (AT_2R; **B**) and endothelial nitric oxide synthase (eNOS; **C**) in cerebral arteries from male and female wild type mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM. **(A)** n=6 for both genders, **(B)** n=14 for both genders and **(C)** n=9 for both genders.

Discussion

The major novel finding of this study is that gender influences cerebrovascular responses to angiotensin II by modulating Nox2-NADPH oxidase-dependent ROS generation. Specifically, this study demonstrated that the production of O_2^- and H_2O_2 in response to angiotensin II is lower in cerebral arteries from female wild type mice compared with males. Furthermore, this lower level of ROS production was associated with selectively smaller constrictor responses to angiotensin II. Both Nox2 protein expression and localisation did not differ between genders. Furthermore, protein expression of SOD1-3, AT_1 and AT_2 receptor or eNOS was similar between genders. However, in Nox2-deficient male mice, O_2^- production and constrictor responses to angiotensin II were reduced, whereas deletion of Nox2 had no effect on either O_2^- production or constrictor responses in female mice. Collectively, these findings demonstrate for the first time that Nox2-NADPH oxidase plays a key role in generating ROS and mediating constrictor responses to angiotensin II in the cerebral circulation of males but not females. Such gender-dependent differences in cerebral artery responses to angiotensin II could conceivably contribute to the lower clinical incidence of cerebrovascular disease observed in humans.

Angiotensin II is a critical mediator of the deleterious effects of hypertension on the cerebral circulation. For example, angiotensin II has been shown to promote cerebrovascular remodelling (Baumbach *et al.*, 2003) and impair nitric oxide (NO·) –dependent relaxation responses (Capone *et al.*, 2010a; Girouard *et al.*, 2006; Kazama *et al.*, 2003). Furthermore, it has recently been reported that angiotensin II impairs NO·–dependent vascular relaxation responses prior to elevating blood pressure (Capone *et al.*, 2010b), suggesting that angiotensin II has direct effects on the cerebral vasculature that are independent of hypertension. Numerous investigators have reported that many of the damaging effects of angiotensin II on the cerebral circulation are mediated by ROS, generated by Nox2-NADPH oxidase (Girouard *et al.*, 2006; Kazama *et al.*, 2004). Epidemiological studies have revealed that the incidence of cerebrovascular diseases such as stroke, are lower in premenopausal females compared with males of similar age (Appelros *et al.*, 2009; Prencipe *et al.*, 1997). Moreover, it has recently been demonstrated that cerebrovascular ROS production is lower in the cerebral circulation of female compared with male rats (Miller *et al.*, 2007b). However, no study has specifically

addressed whether gender influences angiotensin II-induced ROS production in the cerebral circulation. Using the chemiluminescence probe, lucigenin, we found that angiotensin II increases O_2^- production by cerebral arteries from mice of both genders, an effect that was virtually abolished by the flavin antagonist/NADPH oxidase inhibitor, DPI. By contrast, basal O_2^- production by cerebral arteries was similar between genders. The major novel finding of these experiments was that O_2^- production in response to angiotensin II was approximately 70 % lower in female wild type mice than in males. Importantly, these findings were made in the presence of the SOD1 and SOD3 inhibitor, DETCA, thus it seems unlikely that differing rates of O_2^- inactivation by SODs account for these gender differences in detected O_2^- levels. Consistent with this interpretation was our finding that protein expression of all three SOD isoforms in mouse cerebral arteries was similar between genders. It has been previously reported that angiotensin II may increase O_2^- production in the cerebral circulation via activation of COX (Capone *et al.*, 2010a; Haberl *et al.*, 1990). However, in the present study we found that the isoform non-selective COX inhibitor, indomethacin, did not alter angiotensin II-stimulated O_2^- production by mouse cerebral arteries. It is well established that angiotensin II stimulates Nox2-NADPH oxidase-derived ROS production via activation of the AT_1 receptor (Choi *et al.*, 2008; Girouard *et al.*, 2006; Kazama *et al.*, 2004). Furthermore, AT_2 receptor activation has been reported to stimulate the production of NO· by eNOS (Gauthier *et al.*, 2005; Yayama *et al.*, 2006). The reaction of NO· with O_2^- is 3 times faster than the dismutation of O_2^- by SOD (Thomson *et al.*, 1995). Therefore, it is conceivable that the gender-dependent differences in O_2^- levels are due to differential rates of NO· production and hence scavenging of O_2^-. However, we found that protein expression of AT_1 and AT_2 receptors was similar in cerebral arteries from males and females. Thus, it appears unlikely that the gender-related differences in O_2^- levels are attributable to differences in signalling via the AT_1 and/or AT_2 receptors. Furthermore, eNOS protein expression levels did not differ between genders. Nevertheless, we cannot discount the possibility that eNOS activity and hence NO· production, is higher in the cerebral circulation of females. Indeed, it has been reported that isolated basilar arteries from female rats generate more basal NO· (Chrissobolis *et al.*, 2004a). However, it has also been reported that NOS inhibition does not modulate NADPH oxidase-derived O_2^- production by the basilar artery from either male or female rats (Miller *et al.*, 2007b). Taken together, it seems likely that

the gender-dependent difference in angiotensin II-stimulated O_2^- production is due to differences in the rate of O_2^- production.

Under physiological conditions, O_2^- is rapidly metabolised by SODs to form H_2O_2. In contrast to O_2^-, H_2O_2 is highly diffusible and relatively stable. Consequently, H_2O_2 is now thought to be an important ROS molecule for modulating vascular function, including the regulation of vascular tone (Gupte *et al.*, 2009; Miller *et al.*, 2005; Miller *et al.*, 2007b). Therefore, we next tested if gender influenced angiotensin II-stimulated H_2O_2 production in the cerebral circulation. Using Amplex Red fluorescence, we found that angiotensin II-stimulated H_2O_2 production was approximately 85 % lower in arteries from female mice than males. Thus, taken together these experiments reveal that in response to angiotensin II the production of both O_2^- and its metabolite, H_2O_2, are lower in the cerebral circulation of female versus male wild type mice.

In addition to being a strong stimulus of NADPH oxidase activity, angiotensin II is a physiologically relevant vasoconstrictor. Numerous studies of cerebral arteries, including those in humans, have found that angiotensin II elicits a modest vasoconstriction (Amberg *et al.*, 2010; Faraci *et al.*, 2005; Miller *et al.*, 2005; Stenman *et al.*, 2004; Toda *et al.*, 1990; Vincent *et al.*, 2005; Whalley *et al.*, 1985). Therefore, we next tested for any gender difference in the regulation of cerebrovascular tone associated with the differing levels of ROS production in response to angiotensin II. Consistent with the aforementioned findings, we found that application of angiotensin II to pressurised isolated mouse MCA results in constriction in both genders. However, the constrictor response to angiotensin II was approximately 50 % smaller in female versus male mice. These findings in the MCA are consistent with the findings of a previous study of basilar arteries where constrictor responses to angiotensin II were smaller in female versus male mice (Faraci *et al.*, 2005). In the present study, constrictor responses to KPSS were similar between genders. Similarly, previous studies have found that responses of cerebral arteries to high K^+ (Faraci *et al.*, 2005), serotonin (Chrissobolis *et al.*, 2004a) and U46619 (Faraci *et al.*, 2005) are similar between genders. Taken together, these findings suggest that gender-dependent differences in response to angiotensin II found in the present study are likely to be at least somewhat selective for angiotensin II.

The findings of this study are conceptually consistent with a recent study reporting that angiotensin II administration, either acute or chronic, attenuates the increase in cerebral blood flow induced by the NO·-dependent vasodilator, acetylcholine, or whisker stimulation in male, but not female mice (Girouard *et al.*, 2008). This effect of angiotensin II on cerebral artery function occurs independently of changes in blood pressure as chronic phenylepherine treatment, which elevated blood pressure to similar level as angiotensin II, did not impair increases in cerebral blood flow to endothelium dependent stimuli (Girouard *et al.*, 2008). Marked gender-dependent differences in response to angiotensin II have also recently been reported in the cerebral circulation of SOD2 heterozygous deficient ($SOD2^{+/-}$) mice. This study demonstrated that impaired NO·-dependent dilatation was observed in basilar arteries of male, but not female, angiotensin II-treated $SOD2^{+/-}$ mice (Chrissobolis *et al.*, 2010). Therefore, a reduction in vascular antioxidant defence mechanisms leaves cerebral arteries from males, but not females, susceptible to the deleterious effects of angiotensin II, which may be due to greater production of ROS by cerebral arteries from males.

As mentioned, previous studies in cerebral arteries have reported that many of the deleterious effects of angiotensin II on the cerebral circulation, such as increased ROS production and impaired functional hyperaemia, are mediated by Nox2-NADPH oxidase (Girouard *et al.*, 2006; Girouard *et al.*, 2007; Kazama *et al.*, 2004). Therefore, to test for a role for Nox2-NADPH oxidase in the gender-dependent differences in cerebrovascular ROS production and constrictor responses to angiotensin II, we next studied cerebral vessels from $Nox2^{-/-}$ mice. Firstly, we measured angiotensin II-stimulated O_2^- production by cerebral arteries from wild type and $Nox2^{-/-}$ mice using the chemiluminescence probe, L-012. Consistent with our data using lucigenin, O_2^- production was approximately 60 % lower in female versus male wild type mice. Importantly, levels of O_2^- were markedly reduced in male $Nox2^{-/-}$ versus wild type, whereas levels were not altered in female $Nox2^{-/-}$ versus wild type mice. Indeed, O_2^- levels in male $Nox2^{-/-}$ mice were comparable to levels found in female wild type mice. These findings demonstrate that Nox2-NADPH oxidase plays a key role in generating O_2^- in response to angiotensin II in the cerebral circulation of male, but not female mice. We next examined whether Nox2-NADPH oxidase mediates constrictor responses to angiotensin II in MCA from male and female

mice. In female mice, responses of MCA to angiotensin II were similar between wild type and Nox2$^{-/-}$, whereas constrictions were markedly reduced in male Nox2$^{-/-}$ mice such that responses were comparable to those found in female wild type. These findings demonstrate that Nox2-NADPH oxidase plays a key role in 1) generating O_2^-, and its downstream metabolite H_2O_2, and 2) mediating constrictions to angiotensin II in males but not females. These data also provide a mechanistic basis for the marked gender difference in cerebrovascular responses to angiotensin II. It has previously been reported that Nox2 protein is expressed at similar levels in the basilar artery of male and female rats (Miller *et al.*, 2007b). Similarly, we found here that levels of Nox2 protein in the mouse cerebral circulation do not differ between genders. We next examined whether gender influences Nox2 protein localisation in MCA, as differences in the cellular localisation of Nox2 could potentially explain the gender-dependent differences in cerebrovascular responses to angiotensin II. Consistent with previous studies in mouse cerebral arterioles (Kazama *et al.*, 2004), Nox2 immunoreactivity was predominantly localised to endothelial and to a lesser extent, adventitial cells of MCA in male and female mice. Thus, whilst angiotensin II-stimulated Nox2-NADPH oxidase activity appears to be higher in males than females, differing expression levels of Nox2 or its cellular localization apparently do not account for the Nox2-dependent gender differences in responses to angiotensin II. These differences presumably reflect lower Nox2 catalytic activity in females than males (see below).

It is well established that oestrogen exerts numerous protective effects on the cerebral vasculature, such as decreasing cerebral artery tone (Geary *et al.*, 2000) and reducing blood brain barrier permeability (Cipolla *et al.*, 2009a). Moreover, oestrogen has been shown to suppress ROS production in the cerebral circulation. Indeed, a recent study has revealed that cerebral arteries from ovariectomised (OVX) rats generate approximately 3-fold more NADPH oxidase-derived O_2^- compared with both intact and OVX + oestrogen treated female rats (Miller *et al.*, 2007b). Furthermore, OVX rendered female mice susceptible to the impaired cerebrovascular relaxation response induced by angiotensin II administration, an effect that was reversed with oestrogen therapy (Girouard *et al.*, 2008). A more recent study has provided further evidence supporting the notion that the gender-dependent differences in responses to angiotensin II may be due to the actions of

oestrogen on the vasculature. Capone *et al.* (2009) demonstrated that the impairment of functional hyperaemia induced by angiotensin II is dependent on the oestrous cycle of the mouse (Capone *et al.*, 2009). Specifically, when oestrogen levels are high (proestrus and oestrus) the angiotensin II induced impairment of functional hyperaemia was not evident (Capone *et al.*, 2009). In contrast, when oestrogen levels are lower (dioestrus), angiotensin II impaired function hyperaemia to a similar extent as oestrogen receptor inhibition (Capone *et al.*, 2009) and to a level comparable to that seen in male mice (Capone *et al.*, 2010a; Girouard *et al.*, 2008; Girouard *et al.*, 2006). Taken together, these findings clearly suggest that the sexual dimorphism in response to angiotensin II in cerebral arteries may be due to the effects of oestrogen.

Numerous factors may regulate the activity of vascular Nox2, including its association with p47phox, Rac and NADPH (Miller *et al.*, 2006a). As discussed in Chapter 1, the p47phox subunit plays a critical role in the assembly of the active Nox2-NADPH oxidase complex. Electrons are then transferred from the substrate, NADPH, via the Nox catalytic subunit to molecular O_2, generating O_2^-. It has been previously reported that treatment of human macrophages with oestrogen attenuates tumour necrosis factor-α/interferon-γ induced upregulation of p47phox (Sumi *et al.*, 2003). Therefore, it is conceivable that oestrogen-dependent differences in the expression of p47phox could account for the gender dependent differences in response to angiotensin II observed in this study. Although it is unclear if this occurs in vascular tissue, it raises the possibility that the gender-dependent differences in Nox2-NADPH oxidase activity found in the present study are due to relatively lower levels of p47phox in the cerebral circulation of females. In the context of Nox2-NADPH oxidase, PKC is believed to be the key kinase responsible for p47phox phosphorylation and hence Nox2-NADPH oxidase activity (Fontayne *et al.*, 2002). It has been reported that the activity and expression of PKC is lower in the aorta of female rats compared with their male counterparts (Kanashiro *et al.*, 2001). Furthermore, this gender-dependent difference in PKC activity and expression is abrogated following OVX and restored in OVX rats following oestrogen replacement therapy (Kanashiro *et al.*, 2001). Therefore, gender-dependent differences in the activity and/or expression of PKC could explain the difference in cerebral artery Nox2-NADPH oxidase activity that we report in this study. Clearly, future studies are needed to determine if oestrogen has

similar effects on the cerebral circulation and whether the gender-dependent differences in response to angiotensin II are due to the effects of oestrogen on p47phox/PKC.

CHAPTER 4

ROLE OF HYDROGEN PEROXIDE IN ANGIOTENSIN II-INDUCED CONSTRICTION OF CEREBRAL ARTERIES

Introduction

Accumulating evidence suggests that reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) may regulate vascular tone during health and disease. Indeed, it has been shown that ROS can constrict or dilate cerebral arteries from a number of several animal species (Cosentino *et al.*, 1994; Didion *et al.*, 2002; Miller *et al.*, 2005; Miller *et al.*, 2007b; Park *et al.*, 2004; Sobey *et al.*, 1997a). For example, it has been demonstrated that O_2^- can cause contraction of canine basilar arteries to increases in intracellular calcium (Cosentino *et al.*, 1994), whereas in mouse cerebral arteries it has been reported to cause relaxation in response to the NADPH oxidase substrate NADPH (Park *et al.*, 2004). On the other hand, H_2O_2 has been shown to mediate flow-mediated dilatation as well as offset contraction to angiotensin II in rat cerebral arteries (Miller *et al.*, 2005; Paravicini *et al.*, 2006). Taken together, these studies highlight the complex nature of ROS-mediated regulation of cerebrovascular tone and clearly demonstrate the need for further studies to clarify the specific role(s) of ROS in the modulation of vascular tone.

Angiotensin II is the predominant bioactive peptide in the renin-angiotensin system and plays a major role in the regulation of blood pressure. Angiotensin II has been shown to stimulate ROS production (see Chapter 3, Kazama *et al.*, 2004; Miller *et al.*, 2005) and elicit constriction in the cerebral circulation of rats and mice (see Chapter 3, Faraci *et al.*, 2005; Miller *et al.*, 2005; Vincent *et al.*, 2005). Furthermore, it has been demonstrated that angiotensin II-induced constriction of mouse cerebral arteries occurs via activation of the RhoA/Rho-kinase pathway (Faraci *et al.*, 2005). In Chapter 3, we demonstrated that angiotensin II-induced constriction of middle cerebral arteries (MCA) from male Nox2-deficient mice were virtually abolished, suggesting that Nox2-NADPH oxidase-derived ROS play a pivotal role in the signalling cascade that ultimately leads to cerebral vasoconstriction to angiotensin II. However, it is unclear if O_2^- or one of its downstream metabolites such as H_2O_2 mediates the constrictor response to angiotensin II. Therefore, the aim of the present study was to determine the identity of the ROS molecule that mediates vasoconstriction to angiotensin II in the cerebral circulation.

Materials and Methods

All procedures were approved by the institutional animal ethics committee. In total, 46 male C57Bl6/J wild type (8-12 weeks of age; 24.5 ± 0.2 g) mice were studied. All mice were bred at Monash University and housed in an approved animal holding facility.

Vascular Reactivity Studies

MCA from male wild type mice were mounted between two microcannulae in a pressure myograph (Living Systems Instrumentation Inc.) as described in Chapter 2. Following an equilibration period, MCA were exposed to high potassium physiological salt solution to test tissue viability (KPSS; 122.7 mmol/L K^+). After a washout and recovery period, experimental protocols were performed as described below. In all experiments, intraluminal diameter was tracked by a video dimension analyser and constrictor responses expressed as percentage change in intraluminal diameter.

Effect of Tempol and EUK-134 on Angiotensin II-induced Vasoconstriction

Angiotensin II (0.1 nmol/L – 1 µmol/L, 5 min between additions) was added extraluminally to MCA from male wild type mice. Experiments were performed in the absence and presence of the superoxide dismutase (SOD) mimetic tempol (1 mmol/L), the SOD/catalase mimetic EUK-134 (100 µmol/L) or tempol (1 mmol/L) + EUK-134 (100 µmol/L). Arteries were pre-incubated for 10 min with drugs prior to the addition of angiotensin II.

Effect of Exogenous H_2O_2 on Vascular Tone

H_2O_2 (100 µmol/L) was added extraluminally to MCA from male wild type mice. In some experiments, arteries were pre-incubated with EUK-134 (10 min pre-incubation; 100 µmol/L) prior to the addition of H_2O_2.

Effect of Y-276232 on Angiotensin II-induced Vasoconstriction

Angiotensin II (0.1 nmol/L – 1 µmol/L, 5 min between additions) was added extraluminally to MCA from male wild type mice in the absence or presence of the Rho-kinase inhibitor Y-27632 (1 µmol/L; 10 min pre-incubation).

Quantification of H_2O_2 production by Cerebral Arteries

The Amplex Red fluorescence bioassay was used to measure angiotensin II (0.1 µmol/L) -stimulated H_2O_2 production by cerebral arteries (pooled basilar and MCA) from male wild type mice as described in the Chapter 2. Experiments were performed in the presence of either tempol (1 mmol/L) or EUK-134 (100 µmol/L). Basal fluorescence was subtracted from fluorescence in the presence of angiotensin II and H_2O_2 production was normalised to dry tissue weight. H_2O_2 production in the presence of tempol or EUK-134 was expressed as a percentage of H_2O_2 production in control arteries.

Quantification of O_2^- Production by Cerebral Arteries

L-012-enhanced Chemiluminescence

Angiotensin II (0.1 µmol/L) -stimulated O_2^- production by cerebral arteries from male mice was measured using 100 µmol/L L-012-enhanced chemiluminescence as described in Chapter 2. Experiments were performed in the absence and presence of Y-27632 (1 µmol/L; 10 min pre-incubation). Basal counts were subtracted from counts in the presence of angiotensin II from the same animal and O_2^- production was normalized to dry tissue weight.

Drugs

Angiotensin II was purchased from Auspep, Amplex Red fluorescence bioassay kit from Molecular Probes (Invitrogen), EUK-134 from Calbiochem, H_2O_2 from Merck, L-012 from Wako Pure Chemicals (Japan), Y-27632 from Yoshitomi Pharmaceutical Industries Ltd. (Japan) and all other drugs from Sigma. Angiotensin II was dissolved in 0.05 mol/L acetic acid and then diluted in either Krebs-HEPES (L-012 experiments) or Krebs bicarbonate (myograph experiments). EUK-134 was prepared at 100 mmol/L in 100 % DMSO and subsequently diluted in either Krebs-HEPES (Amplex Red Fluorescence Bioassay) or Krebs-

Bicarbonate (myograph experiments). L-012 was prepared at 100 mmol/L in 100 % DMSO and subsequently diluted in Krebs-HEPES. All other drugs were dissolved and diluted in either Krebs-HEPES (L-012/Amplex Red experiments) or Krebs-bicarbonate (myograph experiments). In experiments using EUK-134 or L-012, the concentration of DMSO was ≤0.1 %.

Data Analysis

All results are presented as mean ± SEM. Statistical comparisons were performed using either two-way ANOVA or using a paired or unpaired *t* test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Role of Reactive Oxygen Species in Angiotensin II-induced Constriction of Middle Cerebral Arteries

During the equilibration period vessels did not consistently develop significant myogenic tone. Compared with control responses, constriction of MCA to angiotensin II (0.1 nmol/L – 1 μmol/L) were potentiated in the presence of the SOD mimetic tempol (P<0.05, 1 mmol/L; Figure 1A). In contrast, in either the absence or presence of tempol, the SOD/catalase mimetic EUK-134 (100 μmol/L) virtually abolished constriction to angiotensin II (P<0.05, Figure 1A). EUK-134 treatment had no effect on constrictor responses to KPSS (Figure 1B).

Effect of Tempol and EUK-134 on Angiotensin II-stimulated H_2O_2 Production

In the presence of tempol, angiotensin II-stimulated H_2O_2 production by cerebral arteries was increased by approximately 80 % as compared with levels generated by control arteries (P<0.05, Figure 2A). In contrast, in the presence of EUK-134 (100 μmol/L), angiotensin II-stimulated H_2O_2 production was decreased by approximately 45 % (P<0.05, Figure 2B).

Effect of Exogenous H_2O_2 on Vascular Tone

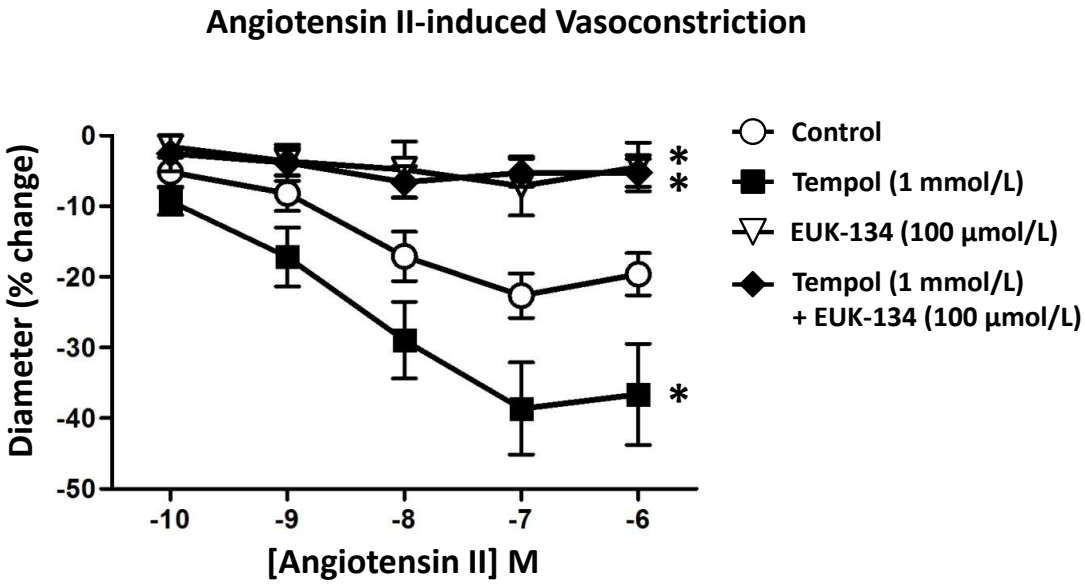
Exogenous H_2O_2 (100 μmol/L) elicited modest constriction of MCA from wild type mice (Figure 3). Constriction to exogenous H_2O_2 were virtually abolished by EUK-134 (100 μmol/L; Figure 3).

Role of Rho-kinase in Cerebral Artery Responses to Angiotensin II

Y-27632 (0.1 μmol/L) had no significant effect on angiotensin II (0.1 μmol/L) –stimulated O_2^- production by cerebral arteries from wild type mice (Figure 4). In the presence of Y-27632 (1 μmol/L), angiotensin II (0.1 nmol/L – 1 μmol/L) –induced constriction of MCA was significantly attenuated (P<0.05, Figure 5A) compared with constriction of control arteries. For example, at 1 μmol/L, angiotensin II constricted control MCA by approximately 20 % compared with approximately 7 % in the presence of Y-27632.

Constriction of MCA to KPSS was similar between control and Y-27632-treated arteries (Figure 5B).

A



B

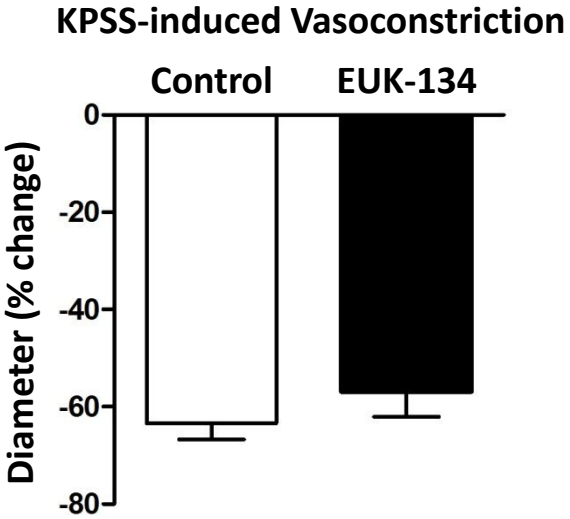


Figure 1. Effect of tempol (1 mmol/L) and EUK-134 (100 µmol/L) on constrictor responses to angiotensin II (0.1 nmol/L – 1 µmol/L) of isolated middle cerebral arteries (MCA) from wild type mice **(A)**. Constrictor responses of isolated MCA to high potassium physiological salt solution (KPSS; 122.7 mmol/L) after treatment with EUK-134 (100 µmol/L) **(B)**. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (**A**, n=8 for control and EUK-134, n=10 for tempol and n=6 for tempol + EUK-134; **B**, n=8 for both groups). ***P*<0.05 vs. control (two-way ANOVA with a Bonferroni multiple comparison post-hoc test).

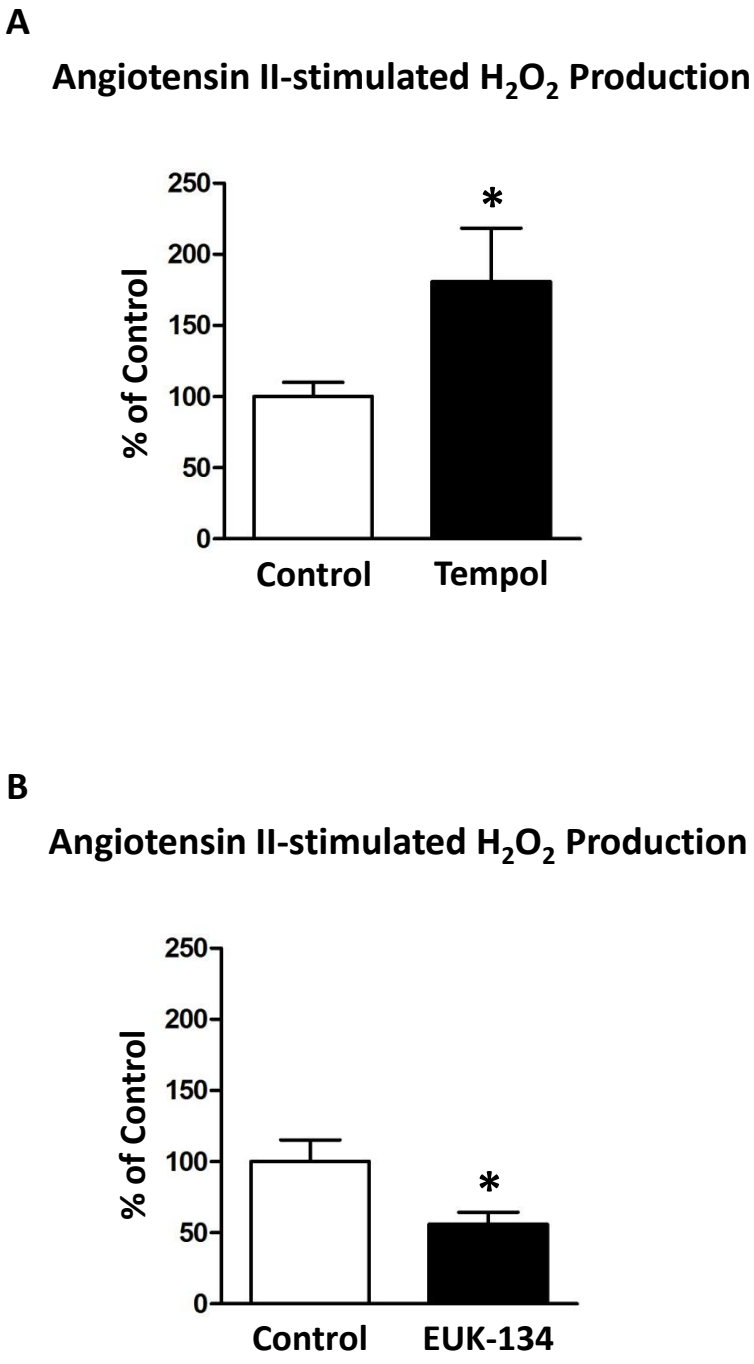


Figure 2. Effect of tempol (1 mmol/L) **(A)** and EUK-134 (100 µmol/L) **(B)** on angiotensin II (0.1 µmol/L)-stimulated H_2O_2 production by cerebral arteries from wild type mice as measured using the Amplex Red Fluorescence Bioassay. H_2O_2 production in the presence of either tempol or EUK-134 was normalised to % of respective control and given as mean ± SEM (**A & B**, n=6 for both groups). ***P*<0.05 vs. control (paired *t* test).

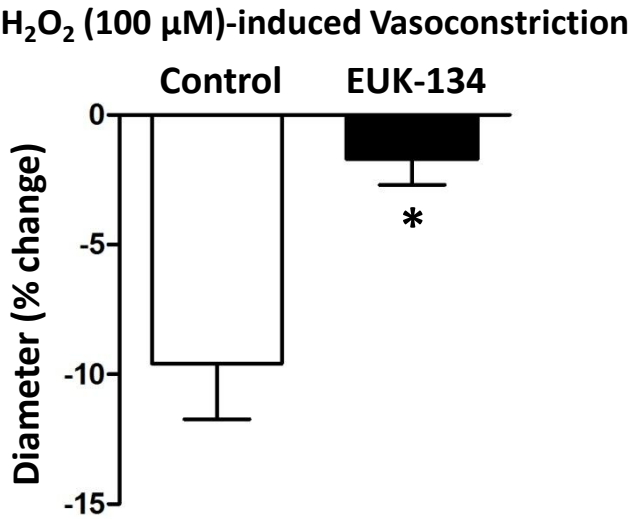


Figure 3. Effect of EUK-134 (100 µmol/L) on constrictor responses of isolated middle cerebral arteries from wild type mice to exogenous H_2O_2 (100 µmol/L). Results are expressed as % change in intraluminal diameter and given as mean ± SEM (n=5 for both groups). *****P<0.05 vs. control (paired t test).

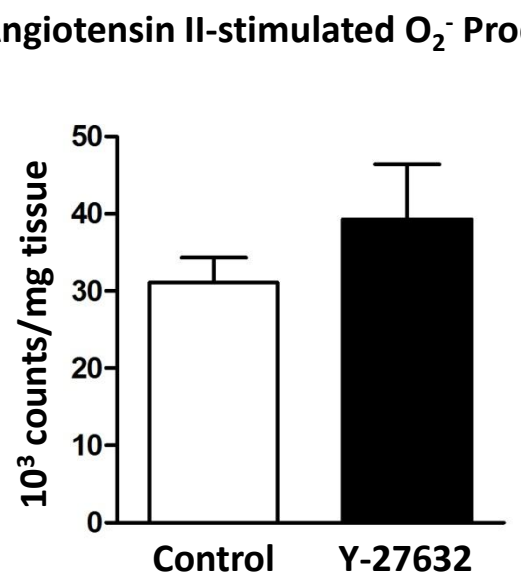
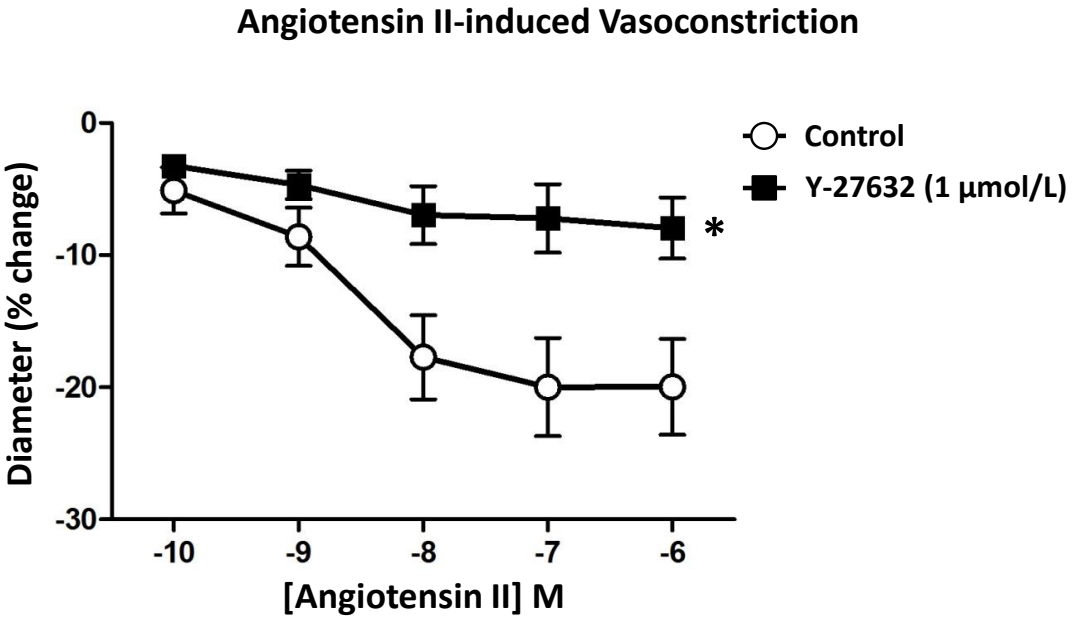


Figure 4. Effect of the Rho-kinase inhibitor Y-27632 (1 µmol/L) on angiotensin II (0.1 µmol/L) -stimulated O_2^- production by cerebral arteries from wild type mice as measured by 100 µmol/L L-012-enhanced chemiluminescence. Results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (n=7 for both groups).

A



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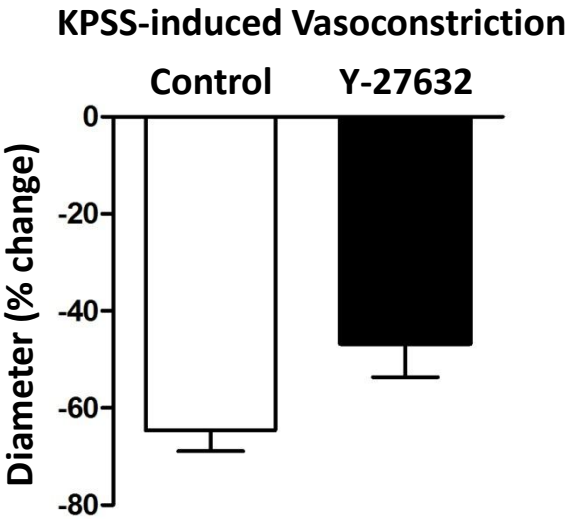


Figure 5. Effect of Y-27632 (1 µmol/L) on constrictor responses to angiotensin II (0.1 nmol/L – 1 µmol/L) of isolated middle cerebral arteries (MCA) from wild type mice **(A)**. Constrictor responses of isolated MCA from wild type mice to high potassium physiological salt solution (KPSS; 122.7 mmol/L) after treatment with Y-27632 (1 µmol/L) **(B)**. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (**A** and **B**, n=5 both groups). ***P*<0.05 vs. control (two-way ANOVA with a Bonferroni multiple comparison post-hoc test).

Discussion

The major finding of this study is that in the cerebral circulation of male mice, vasoconstriction to angiotensin II are mediated by the ROS, H_2O_2. Specifically, we show that the SOD mimetic, tempol, potentiated constriction of MCA to angiotensin II, whereas the SOD/catalase mimetic, EUK-134, virtually abolished constriction. Furthermore, we demonstrate that constriction to angiotensin II involves the activation of the RhoA/Rho-kinase pathway. Collectively, these findings suggest that in response to angiotensin II, H_2O_2 - primarily generated by Nox2-NADPH oxidase (see Chapter 3) - activates the RhoA/Rho-kinase pathway resulting in constriction of cerebral arteries.

It is well documented that O_2^- and H_2O_2 can modulate cerebrovascular tone (Miller *et al.*, 2006a). The modulation of cerebrovascular tone by ROS is complex and is dependent on the identity of the ROS molecule, the concentration and the animal species under investigation. For example, O_2^- has been shown to elicit dilatation of mouse cerebral arterioles in response to a number of stimuli including bradykinin and NADPH (substrate for NADPH oxidase) (Niwa *et al.*, 2001; Park *et al.*, 2004). Furthermore, it has been demonstrated that O_2^- can mediate both relaxation and contraction of rabbit cerebral arteries to NADH (NADPH oxidase substrate) (Didion *et al.*, 2002), and contraction of canine cerebral arteries to the calcium ionophore A23187 (Cosentino *et al.*, 1994). Due to its ability to diffuse across cell membranes and its longer half life, H_2O_2 may be a particularly important ROS signalling molecule. Like O_2^-, H_2O_2 has also been reported to mediate both contraction and relaxation of cerebral and non-cerebral arteries (see below). In Chapter 3, we demonstrated a key role for Nox2-NADPH oxidase in the gender-dependent differences in cerebral artery responses to angiotensin II. In addition, we showed that angiotensin II-induced constriction of MCA from male $Nox2^{-/-}$ mice was virtually abolished compared with wild type mice (see Chapter 3, Figure 6A). These findings indicate that ROS mediate constriction to angiotensin II in the cerebral circulation. However, it is unclear if O_2^- or one of its downstream metabolites such as H_2O_2 mediates angiotensin II-induced constriction of cerebral arteries. Therefore, we sought to determine the identity of the ROS molecule that was mediating constriction of cerebral arteries to angiotensin II. In the present study, we found that the SOD mimetic, tempol, potentiated constriction of the MCA to angiotensin II and increased angiotensin

II-stimulated H_2O_2 levels (measured using the Amplex Red Fluorescence Bioassay). These findings raise the possibility that H_2O_2 mediates angiotensin II-induced constriction of the mouse MCA. Indeed, the SOD/catalase mimetic, EUK-134, virtually abolished angiotensin II-induced constriction and decreased angiotensin II-stimulated H_2O_2 levels (measured using the Amplex Red Fluorescence Bioassay). Importantly, EUK-134 had no effect on constrictor responses to KPSS, suggesting EUK-134 does not alter the ability of MCA to constrict. Moreover, we also found that exogenous application of H_2O_2 to mouse MCA elicited a sustained constriction, which was attenuated by EUK-134. Thus, taken together these findings demonstrate, for the first time, that H_2O_2 mediates constriction to angiotensin II in the mouse cerebral circulation.

As mentioned, previous studies have reported that H_2O_2 can modulate vascular tone. Consistent with the findings of the present study, exogenous H_2O_2 has been demonstrated to contract cerebral arteries from monkeys (Toda *et al.*, 1992). In addition, H_2O_2-mediated contraction has also been observed in the aorta, mesenteric and carotid arteries of the mouse (Ardanaz *et al.*, 2007; Ardanaz *et al.*, 2008; Gupte *et al.*, 2009). Interestingly, and consistent with our findings in Chapter 3, a recent study found that Nox2-NADPH oxidase-derived ROS mediate contraction of aortae to phorbol-12, 13-dibutyrate, a Nox2-NADPH oxidase activator (Gupte *et al.*, 2009). However, in contrast with these aforementioned studies, it has been reported that H_2O_2 dilates mouse and rat cerebral arteries in response to arachidonic acid (Modrick *et al.*, 2009), bradykinin (Sobey *et al.*, 1997a) and flow (Drouin *et al.*, 2009; Paravicini *et al.*, 2006). Furthermore, previous studies have reported that NADPH oxidase-derived H_2O_2 offsets contraction of the rat basilar (Miller *et al.*, 2005) and human coronary artery (Schuijt *et al.*, 2003) to angiotensin II. Moreover, exogenous application of H_2O_2 to the rat basilar artery induced a small contraction followed by sustained relaxation (Miller *et al.*, 2005). These aforementioned findings indicate that H_2O_2 has varying effects on vascular tone depending on the species, artery and stimuli under investigation. Clearly, the reason(s) for these differences in vascular responses to H_2O_2, and especially what role H_2O_2 plays in cerebral responses to angiotensin II in humans, requires further investigation.

It is well established that angiotensin II elicits constriction of cerebral arteries via

activation of the AT_1 receptor. Indeed, it has been demonstrated that constriction to angiotensin II is abolished in AT_{1A} receptor-deficient mice (Faraci *et al.*, 2005) and in cerebral arteries treated with the AT_1 receptor antagonist telmisartan (Vincent *et al.*, 2005). Angiotensin II-induced contraction of smooth muscle occurs via AT_1 receptor mediated activation of phospholipase C, resulting in inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) production. IP_3 and DAG regulate two distinct pathways however, both ultimately result in smooth muscle contraction via activation of a number of protein kinases, such as myosin light chain kinase and Rho-kinase (Hilgers *et al.*, 2005). Furthermore, it has previously been shown that angiotensin II-induced constriction of basilar arteries is abolished in the presence of the Rho-kinase inhibitor, Y-27632 (Faraci *et al.*, 2005). Therefore, we next tested if angiotensin II-induced constriction of MCA was dependent on Rho-kinase. In the presence of the Rho-kinase inhibitor Y-27632, vasoconstriction to angiotensin II was significantly attenuated. To minimise the possibility of Y-27632 inhibiting other protein kinases such as protein kinase C (PKC), we used a low concentration of Y-27632 that is unlikely to inhibit PKC activity (Davies *et al.*, 2000). Therefore, the inhibitory effect of Y-27632 on angiotensin II-induced constriction of MCA is likely to be due to selective inhibition of Rho-kinase. In the present study, we found that angiotensin II-stimulated O_2^- production by cerebral arteries was not affected by Y-27632, suggesting that Rho-kinase activation is downstream of ROS generation. Thus, taken together with our functional data, these findings suggest that H_2O_2 activates the RhoA/Rho-kinase pathway, either indirectly or directly, to elicit constriction of MCA. To the best of our knowledge, there is no evidence that Rho-kinase is directly redox-sensitive and therefore, it is unlikely to be directly activated by H_2O_2. However, the activity of the small G-protein RhoA, the principal activator of Rho-kinase, is believed to be redox-sensitive and thus may be modulated by ROS. Indeed, it has been reported that both O_2^- and t-butyl hydroperoxide (a stable analogue of H_2O_2) can activate RhoA (Aghajanian *et al.*, 2009; Broughton *et al.*, 2009; Jernigan *et al.*, 2008; Jin *et al.*, 2004). Specifically, it has been demonstrated that oxidation of two cysteine residues in the phosphoryl binding loop of RhoA by t-butyl hydroperoxide results in the exchange of guanosine diphsophate (GDP) for guanosine triphosphate (GTP), and activation of RhoA (Aghajanian *et al.*, 2009). Once active, RhoA then translocates to the cell membrane where it binds to and activates Rho-kinase (Leung *et al.*, 1995). This may result in signalling via the well established

pathway of Rho-kinase dependent inhibition of myosin light chain phosphatase (MLCP), thus preventing the de-phosphorylation of myosin (Jernigan *et al.*, 2008; Jin *et al.*, 2004; Kimura *et al.*, 1996). However, it is also possible that Rho-kinase mediated constriction may be due to direct phosphorylation of myosin light chain (MLC) by Rho-kinase (Katoh *et al.*, 2001) or by Rho-kinase mediated activation of other MLC kinases such as zipper-interacting protein kinase, which in turn phosphorylates MLC causing smooth muscle contraction (Hagerty *et al.*, 2007). Currently, it is unknown if H_2O_2 activates Rho-kinase through activation of RhoA (as discussed above) or via other mechanisms. Nevertheless, the results of the present study indicate that H_2O_2 plays a pivotal role in this signalling cascade. Clearly, future studies are needed to determine the mechanisms by which Nox2-NADPH oxidase-derived H_2O_2 activates the RhoA/Rho-kinase pathway to elicit constriction of cerebral arteries in response to angiotensin II.

CHAPTER 5

AUGMENTED SUPEROXIDE PRODUCTION BY NOX2-NADPH OXIDASE CAUSES CEREBRAL ARTERY DYSFUNCTION DURING HYPERCHOLESTEROLAEMIA

Introduction

NADPH oxidases are an important source of superoxide (O_2^-) in the cerebral circulation under both physiological and pathophysiological conditions (Jackman *et al.*, 2009b; Kazama *et al.*, 2004; Miller *et al.*, 2005; Miller *et al.*, 2007b; Park *et al.*, 2008). As discussed in Chapter 1, these enzymes comprise two membrane-bound subunits, Nox (catalytic subunit) and p22phox, up to three cytoplasmic subunits (p47phox/NoxO1, p67phox/NoxA1 and possibly p40phox) and a small G-protein, Rac (Miller *et al.*, 2006a; Selemidis *et al.*, 2008). NADPH oxidases generate O_2^- by transferring electrons from the substrate, NADPH, to molecular oxygen via the Nox catalytic subunit. Cerebral arteries express the Nox1-, Nox2- and Nox4-containing isoforms of NADPH oxidase (Ago *et al.*, 2005; Miller *et al.*, 2007b). In Chapter 3, it was demonstrated that Nox2-NADPH oxidase contributes to angiotensin II-stimulated O_2^- production by cerebral arteries from male mice. Furthermore, a recent study from our lab has demonstrated that Nox2-NADPH oxidase contributes to NADPH-stimulated O_2^- production in the cerebral circulation of mice under physiological conditions (Miller *et al.*, 2009). Moreover, excessive production of O_2^- by Nox2-NADPH oxidase has been linked with cerebral artery dysfunction in experimental models of hypertension (Girouard *et al.*, 2006), Alzheimer's disease (Park *et al.*, 2008) and aging (Park *et al.*, 2007). Thus, the findings of these studies suggest that Nox2-NADPH oxidase may be an important source O_2^- in the cerebral circulation during disease.

It is well established that hypercholesterolaemia is a risk factor for coronary and peripheral vascular disease. In contrast, the relationship between cholesterol levels and stroke incidence has historically been controversial (Demchuk *et al.*, 1999; Landau, 1999). Indeed, several large cohort studies have reported little or no correlation between total cholesterol levels and stroke incidence (Kagan *et al.*, 1980; Wannamethee *et al.*, 2000), whereas others have reported a positive correlation (Iso *et al.*, 1989; Kannel *et al.*, 1965). More recent studies suggest a positive correlation between elevated plasma levels of low density lipoprotein (LDL) cholesterol and increased stroke incidence (Amarenco *et al.*, 2006; Imamura *et al.*, 2009). Nevertheless, in some, but not all, studies in animal models of hypercholesterolaemia, it has been reported that high cholesterol levels attenuate nitric oxide (NO·) -dependent relaxation responses in the cerebral circulation, which is a

classic hallmark of endothelial dysfunction (Kitagawa *et al.*, 1994; Kitayama *et al.*, 2007; Rossitch *et al.*, 1991; Simonsen *et al.*, 1991; Stewart-Lee *et al.*, 1991; Yamashiro *et al.*, 2010). Importantly, evidence suggests that increased O_2^- production by cerebral vascular NADPH oxidases during hypercholesterolaemia may play a role in compromised NO· function (Kitayama *et al.*, 2007). However, the contribution of Nox2-NADPH oxidase-derived O_2^- in NO· dysfunction associated with hypercholesterolaemia has not been evaluated. Therefore, in the present study we sought to test whether excessive production of O_2^- by Nox2-NADPH oxidase occurs in the cerebral circulation of hypercholesterolaemic apolipoprotein E-deficient ($ApoE^{-/-}$) mice and whether this causes decreased NO· function.

Methods

All procedures were approved by the institutional animal ethics committee. Genetically related wild type, Nox2-deficient ($Nox2^{-/-}$), $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ colonies were generated as described in Chapter 2. Briefly, $Nox2^{-/-}$ mice were obtained from Ozgene (Australia), wild type and $ApoE^{-/-}$ mice were from the Animal Resources Centre (Australia). $Nox2^{-/-}$ mice were bred with $ApoE^{-/-}$ mice to generate a $Nox2^{-/-}/ApoE^{-/-}$ double knockout colony, and a genetically-related $ApoE^{-/-}$ single knockout colony. All mice studied were male and were of the C57Bl/6J background. Genotypes were determined by PCR amplification of tail DNA. From five weeks of age, mice were maintained on a high fat diet (21 % fat, 0.15 % cholesterol; Specialty Feeds, Australia) for 7-14 weeks. p47phox-deficient ($p47phox^{-/-}$) mice were originally obtained from Taconic Labs (USA). All mice were bred at Monash University and housed in an approved animal holding facility. In total, 56 C57Bl6/J wild type (39.6 ± 1.2 g), 17 $Nox2^{-/-}$ (32.7 ± 1.0 g), 65 $ApoE^{-/-}$ (34.9 ± 2.2 g), 55 $Nox2^{-/-}/ApoE^{-/-}$ (34.0 ± 1.2 g) and 7 $p47phox^{-/-}$ (25.3 ± 0.8 g) mice were studied.

Measurement of Total Plasma Cholesterol Levels

Blood was collected into a heparinised tube and centrifuged at 4,000 x g (4°C) for 10 min. High density lipoprotein (HDL), LDL and total plasma cholesterol levels were then determined using a Roche MODULAR$^{(TM)}$ 917 (Roche Diagnostics, USA) enzymatic colorimetric assay.

Histological studies

Cross Sectional Oil Red O Staining

Oil red O staining was used to evaluate middle cerebral arteries (MCA) and aorta for the presence of atherosclerotic lesions. MCA from wild type, $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ mice were prepared for histological analysis as described in Chapter 2. Aortae from wild type and $ApoE^{-/-}$ mice were dissected, cleared of adherent fat and embedded in OCT Tissue-Tek (Sakura Finetek, U.S.A.). MCA and aortae were sectioned (10 µm) and incubated for 60 min with oil red O (0.5 % in 60 % isopropyl alcohol). Excess stain was removed with 60 % isopropyl alcohol and sections were then counterstained with 25 % haematoxylin. Four sections (approximately 200 µm apart from each other along the length of the artery) per animal were viewed and digitised using an Olympus AX70 microscope equipped with a

40× oil immersion objective lens (for MCA) or 10x objective lens (for aorta) and a colour DP70 Peltier cooled digital camera (Olympus, Japan).

Haematoxylin & Eosin Staining

In addition to oil red O staining, haematoxylin and eosin staining was used to further assess MCA for the presence of atherosclerotic lesions. MCA from wild type, $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ mice were prepared for histological analysis as described in Chapter 2. Four sections (approximately 200 µm apart from each other along the length of the artery) per animal were viewed and digitised using an Olympus BH-2 (Olympus, Japan) microscope equipped with a 40× objective lens and a colour XC-77CE digital camera (Sony, Japan).

En Face Oil Red O Staining

En Face oil red O staining was used to quantify atherosclerotic lesion burden in aorta from wild type and $ApoE^{-/-}$ mice. Aortae were dissected in their entirety and cut open longitudinally along the ventral surface and incubated with oil red O. Excess stain was removed with 60 % isopropyl alcohol and *en face* images of each aortic segment were captured using a Moticam-480P Digital Camera and quantified using Motic Images Plus 2.0 software. Lesion area was expressed as a percentage of the total luminal surface area.

Quantification of O_2^- Production by Cerebral Arteries

Basal O_2^- production by cerebral arteries (pooled basilar and MCA) from wild type, $Nox2^{-/-}$, $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ mice was measured by 100 µmol/L L-012-enhanced chemiluminescence as described in Chapter 2. Background counts were subtracted and O_2^- production was normalised to dry tissue weight. In some experiments, basal O_2^- production by cerebral arteries from $ApoE^{-/-}$ mice was assessed in the presence of the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L; 30 min pre-incubation).

Assessment of NO· Function in Cerebral Arteries

MCA from wild type, $Nox2^{-/-}$, $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ mice were mounted between two microcannulae in a pressure myograph (Living Systems Instrumentation Inc., USA) as

described in Chapter 2. NO· function was assessed via the constrictor response (measured from baseline diameter) to the NOS inhibitor L-NAME (100 μmol/L; 30 min incubation). Constrictor responses were recorded once they reached a steady level (after approximately 30 min). In MCA from a separate group of wild type and $ApoE^{-/-}$ mice, constrictor responses to L-NAME were assessed in the presence of the O_2^- scavenger, tempol (1 mmol/L; 10 min pre-incubation). Following equilibration and on completion of the experimental protocol, arteries were exposed to a high potassium physiological salt solution (KPSS) containing 122.7 mmol/L K^+ (equimolar replacement of NaCl with KCl). Functional studies testing the effect of tempol on constrictor responses of MCA to L-NAME were performed on the same day.

Western Blotting

Experiments were carried out using pooled basilar arteries and MCA. Protein expression of Nox2 (antibody concentration, 1:1000), p47phox (1:100), superoxide dismutase (SOD) isoforms (SOD1, 1:4000; SOD2, 1:1000; SOD3, 1:1000), endothelial NOS (1:1000), monocyte chemoattractant protein 1 (MCP-1; 1:500) and vascular cell adhesion molecule 1 (VCAM-1; 1:250) was measured in cerebral arteries from wild type, $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ mice using Western blotting as described in Chapter 2. In all experiments, relative protein intensity was normalised to the corresponding band for β-actin (1:2000) as described in Chapter 2.

Antibodies & Drugs

Anti-Nox2 and anti-eNOS mouse monoclonal antibodies were purchased from BD Biosciences (U.S.A). Anti-p47phox mouse monoclonal and anti-VCAM-1 rat monoclonal antibodies from Santa Cruz Biotechnology (U.S.A.). Anti-SOD1 rabbit polyclonal antibody was purchased from Stressgen (Canada), anti-SOD2 anti-SOD3 rabbit polyclonal antibodies from Upstate Cell Signalling Solutions (U.S.A.), anti-MCP-1 mouse monoclonal antibody from GeneScript (U.S.A.) and anti-β-actin mouse/rabbit polyclonal antibodies from Cell Signaling Technology (U.S.A.).

L-012 was purchased from Wako Pure Chemicals (Japan) and all other drugs were from Sigma. L-012 was prepared in 100 mmol/L in 100 % dimethyl sulfoxide (DMSO) and

diluted in Krebs-HEPES solution, such that the final concentration of DMSO was ≤0.1 %. L-NAME and tempol were dissolved in Krebs-bicarbonate solution.

Data Analysis

All results are presented as mean ± SEM. Statistical comparisons were performed using one-way ANOVA with a Bonferroni multiple comparison post-hoc test, or using unpaired *t* test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Total Plasma Cholesterol Levels

Total plasma cholesterol levels were substantially higher in ApoE$^{-/-}$ (11.0 $\pm$ 0.9 mmol/L, P<0.05) and Nox2$^{-/-}$/ApoE$^{-/-}$ (9.9 $\pm$ 0.5 mmol/L, P<0.05) mice than in wild type mice (1.4 $\pm$ 0.1 mmol/L) (Figure 1). The ratio of HDL:LDL cholesterol was similar between ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice (ApoE$^{-/-}$, 0.42 $\pm$ 0.05 vs. Nox2$^{-/-}$/ApoE$^{-/-}$, 0.39 $\pm$ 0.03, P>0.05, n=14 for ApoE$^{-/-}$ and n=17 for Nox2$^{-/-}$/ApoE$^{-/-}$).

Vascular Morphology of Middle Cerebral Arteries and Aorta

Using either oil red O (Figure 2A-C) or haematoxylin and eosin (Figure 2D-F) staining, no lesions or fatty deposits were detected in MCA of wild type, ApoE$^{-/-}$ or Nox2$^{-/-}$/ApoE$^{-/-}$ mice. In contrast, atherosclerotic lesions were found in oil red O stained cross sections of aortae from ApoE$^{-/-}$ but not wild type mice (Figure 3A & B). Using *en face* oil red O staining, atherosclerotic lesions covered approximately 9 % of the total aortic surface in ApoE$^{-/-}$ mice (n=13; Figure 3D) but were undetectable in wild type aorta (n=13; Figure 3B). Previously, our lab has shown that atherosclerotic lesions are significantly reduced at 12-19 weeks of age in aortae of Nox2$^{-/-}$/ApoE$^{-/-}$ mice compared with ApoE$^{-/-}$ mice (Judkins *et al.*, 2009).

Basal O_2^- Production by Cerebral Arteries

Basal O_2^- production by cerebral arteries was similar in wild type and Nox2$^{-/-}$ (Figure 4). However, basal O_2^- production by cerebral arteries from ApoE$^{-/-}$ mice was approximately 2-fold greater than levels generated by arteries from wild type mice (Figure 4, P<0.05). By contrast, in Nox2$^{-/-}$/ApoE$^{-/-}$ mice, cerebral artery O_2^- production was not elevated (Figure 4, P<0.05). The NOS inhibitor L-NAME had no significant effect on O_2^- production by cerebral arteries from ApoE$^{-/-}$ mice (Figure 5).

Cerebral Artery NO· Function

During the equilibration period vessels did not consistently develop significant myogenic tone. Furthermore, baseline diameters of MCA were similar between genotypes (Table 1). The magnitude of L-NAME-induced constriction (measured from baseline diameter) of MCA from ApoE$^{-/-}$ mice was less than 50 % of that in wild type (Figure 6A, P<0.05). By

contrast, constriction to L-NAME in Nox2$^{-/-}$/ApoE$^{-/-}$ mice was comparable to that in wild type mice (Figure 6A). Constrictor responses to KPSS (also measured from baseline diameter) were similar between all genotypes (Figure 6B). In the presence of the O_2^- scavenger, tempol (1 mmol/L), constrictor responses to L-NAME in MCA from ApoE$^{-/-}$ mice were comparable to those of wild type vessels (Figure 7A). Responses to KPSS were similar between MCA from wild type and ApoE$^{-/-}$ mice following treatment with tempol (Figure 7B).

	Wild type	**Nox2$^{-/-}$**	**ApoE$^{-/-}$**	**Nox2$^{-/-}$/ApoE$^{-/-}$**
Diameter (μm)	116 ± 6 (6)	124 ± 4 (6)	107 ± 5 (6)	125 ± 5 (6)

Table 1. Intraluminal diameters of isolated middle cerebral arteries from male wild type, Nox2$^{-/-}$, ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice (n numbers given in brackets).

Cerebral Artery Protein Expression of Nox2-NADPH Oxidase Subunits

Cerebral artery expression of the Nox2 catalytic subunit (~58 kDa) was found to be similar between wild type and ApoE$^{-/-}$ mice (Figure 8). At ~58kDa, there was no Nox2 immunoreactive band in cerebral artery homogenates from either Nox2$^{-/-}$ or Nox2$^{-/-}$/ApoE$^{-/-}$ mice (Figure 8). Nox2 immunoreactivity was also observed at higher molecular weights (between approximately 65 kDa and 110 kDa) however, these bands were not observed consistently and were also observed in cerebral artery homogenates from Nox2$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice (Figure 8, top). In ApoE$^{-/-}$ mice, cerebral artery expression of the Nox2-NADPH oxidase organiser subunit p47phox was approximately 2-fold higher versus wild type mice (Figure 9; $P<0.05$) but was not significantly different to levels found in Nox2$^{-/-}$/ApoE$^{-/-}$ mice (Figure 9). At ~47kDa, there was no p47phox immunoreactive band in cerebral artery homogenates from p47phox$^{-/-}$ mice (Figure 9).

Cerebral Artery Protein Expression of SOD, eNOS, MCP-1 and VCAM-1

Cerebral artery protein expression of SOD isoforms (1, 2 and 3) and eNOS were similar in wild type, ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice (Figure 10A-D). Protein expression of the pro-inflammatory chemokine, MCP-1, was approximately 2-fold higher in cerebral arteries from ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice compared with wild types (Figure 11A;

P<0.05). Cerebral artery protein expression of the adhesion molecule, VCAM-1, was similar in cerebral arteries from wild type, $ApoE^{-/-}$ and $Nox2^{-/-}/ApoE^{-/-}$ mice (Figure 11B).

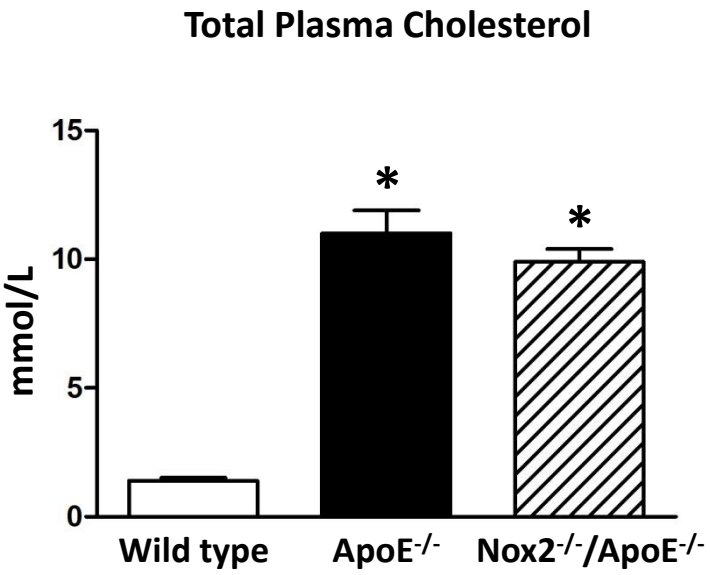


Figure 1. Total plasma cholesterol levels in wild type, apolipoprotein E-deficient ($ApoE^{-/-}$) and Nox2-deficient ($Nox2^{-/-}$)/$ApoE^{-/-}$ mice. All results are expressed in mmol of cholesterol per litre and given as mean ± SEM (n=7 for wild type; n=14 for $ApoE^{-/-}$; n=17 for $Nox2^{-/-}$/$ApoE^{-/-}$). *****P<0.05 vs. wild type (one-way ANOVA with a Bonferroni multiple comparison post-hoc test).

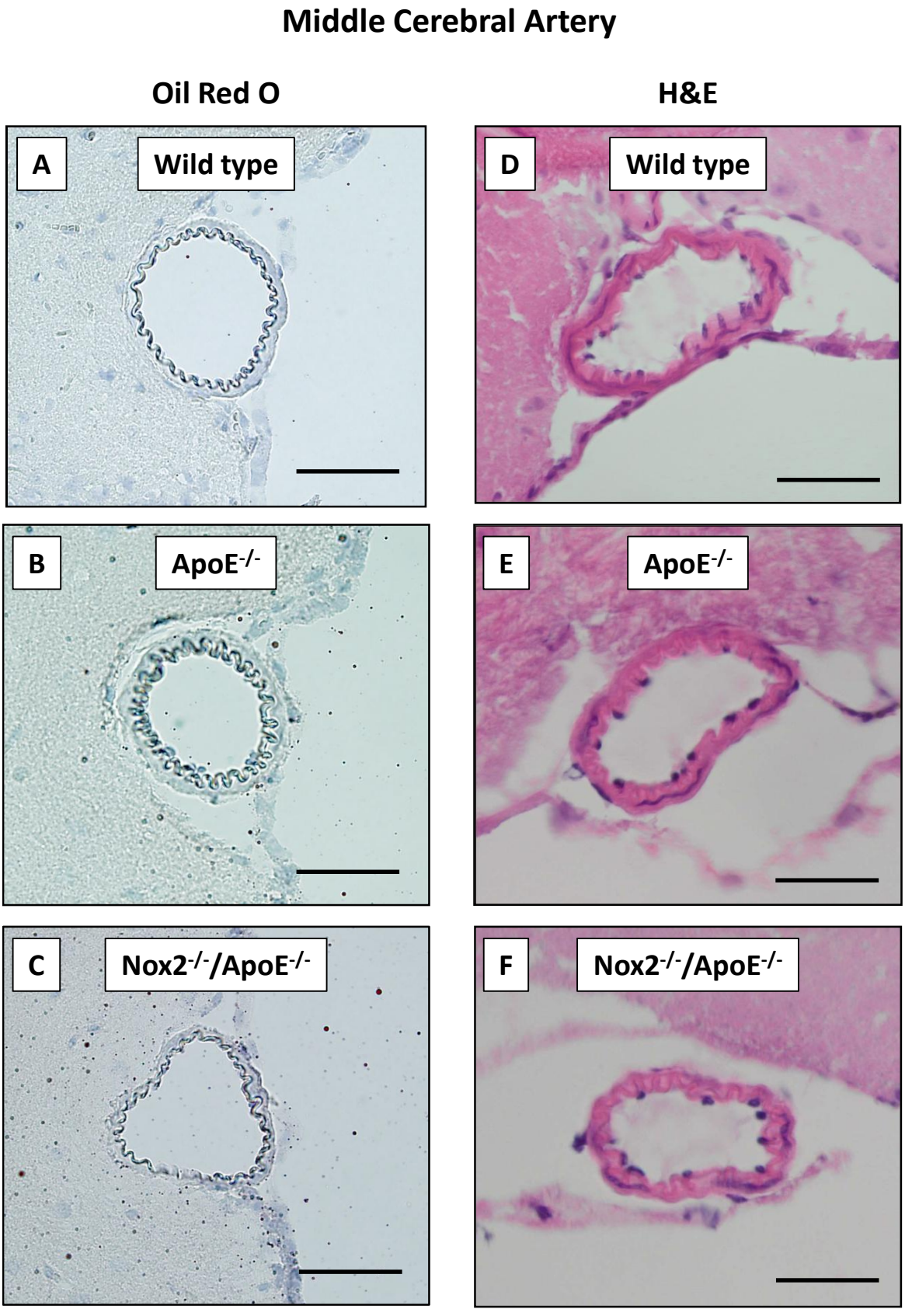

Middle Cerebral Artery
Oil Red O
H&E
A
Wild type
D
Wild type
B
ApoE-/-
E
ApoE-/-
C
Nox2-/-/ApoE-/-
F
Nox2-/-/ApoE-/-

Figure 2. Representative photomicrographs showing sections of the middle cerebral artery from wild type, apolipoprotein E-deficient ($ApoE^{-/-}$) and Nox2-deficient ($Nox2^{-/-}$)/$ApoE^{-/-}$ mice following Oil Red O **(A-C)** and haematoxylin & eosin **(D-F)** staining. Magnification 400×, scale bar=40 µm. Representative of n=3 for all groups.

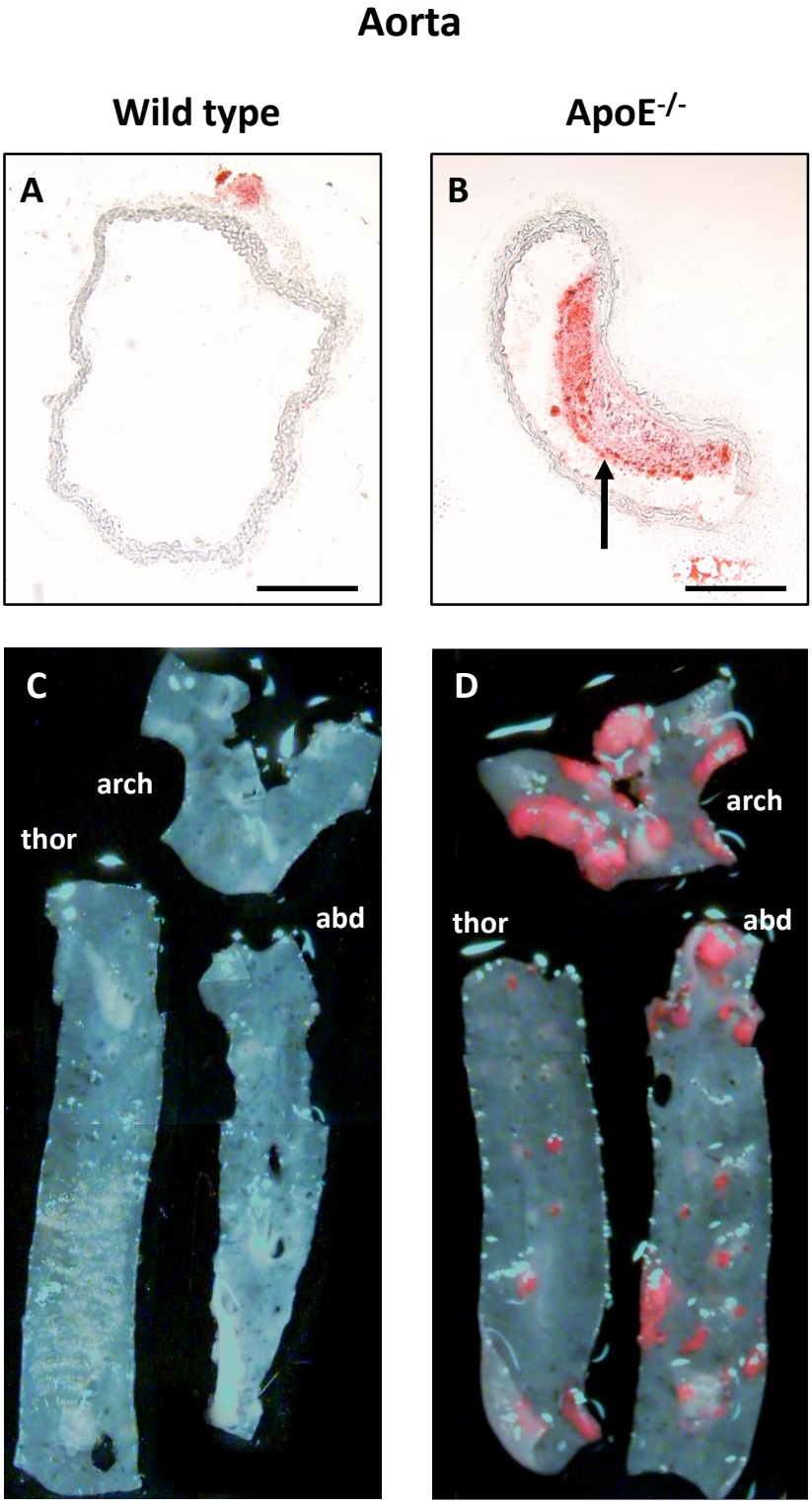

Aorta
Wild type
ApoE-/-
A
B
C
arch
thor
abd
D
arch
thor
abd

Figure 3. Representative photomicrographs of cross sections of the abdominal aorta **(A & B)** and representative *en face* images of the aorta divided into the aortic arch (arch), thoracic (thor) and abdominal (abd) aorta **(C & D)** from wild type and apolipoprotein E-deficient ($ApoE^{-/-}$) mice following oil Red O staining. Red staining and arrow indicates atherosclerotic lesions. Magnification 100×, scale bar=200µm, representative of n=3 for both groups **(A & B)**, representative of n=13 for both groups **(C & D)**.

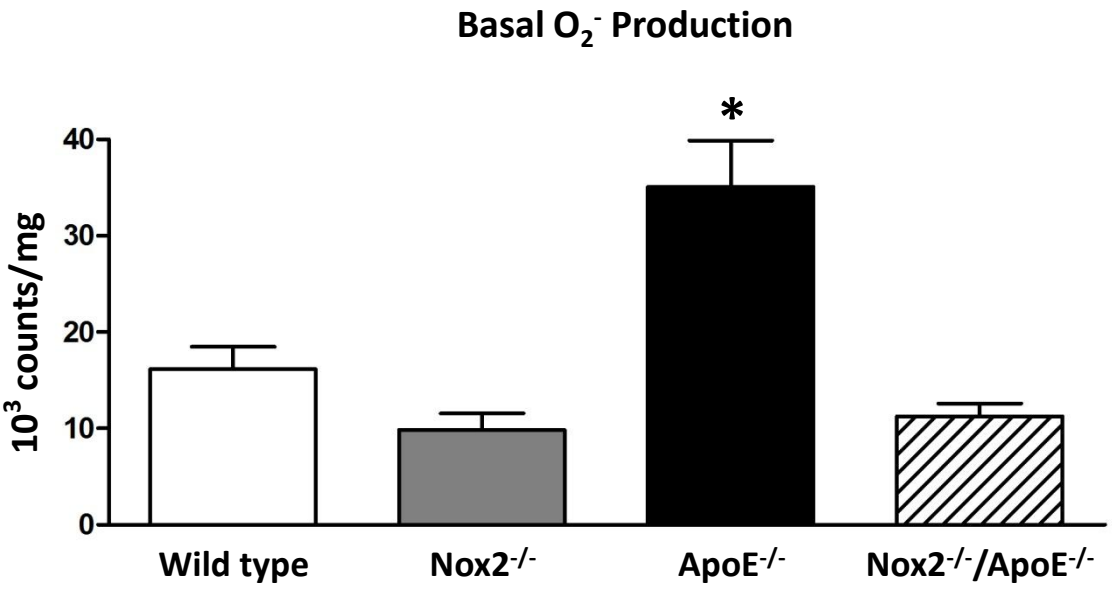


Figure 4. Basal O_2^- production by cerebral arteries from wild type, Nox2-deficient ($Nox2^{-/-}$), apolipoprotein E-deficient ($ApoE^{-/-}$) and $Nox2^{-/-}/ApoE^{-/-}$ mice as measured by 100 µmol/L L-012-enhanced chemiluminescence. All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (n=7 for wild type; n=11 for $Nox2^{-/-}$; n=14 for $ApoE^{-/-}$; n=17 for $Nox2^{-/-}/ApoE^{-/-}$). ***P*<0.05 vs. all other groups (one-way ANOVA with a Bonferroni multiple comparison post-hoc test).

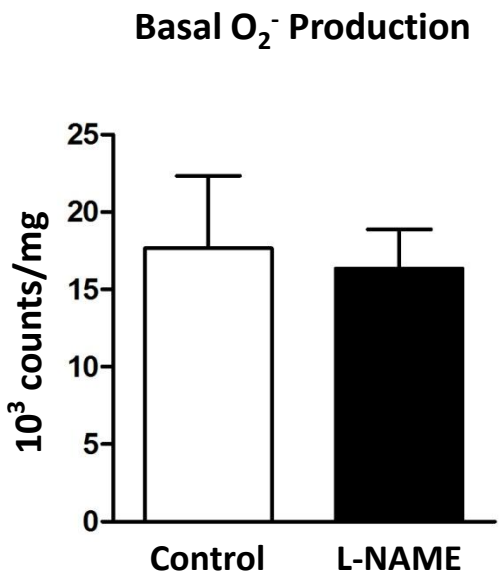


Figure 5. The effect of the NOS inhibitor L-NAME (100 µmol/L) on basal O_2^- production by cerebral arteries from apolipoprotein E-deficient ($ApoE^{-/-}$) mice as measured by 100 µmol/L L-012-enhanced chemiluminescence. All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (n=5 for both groups).

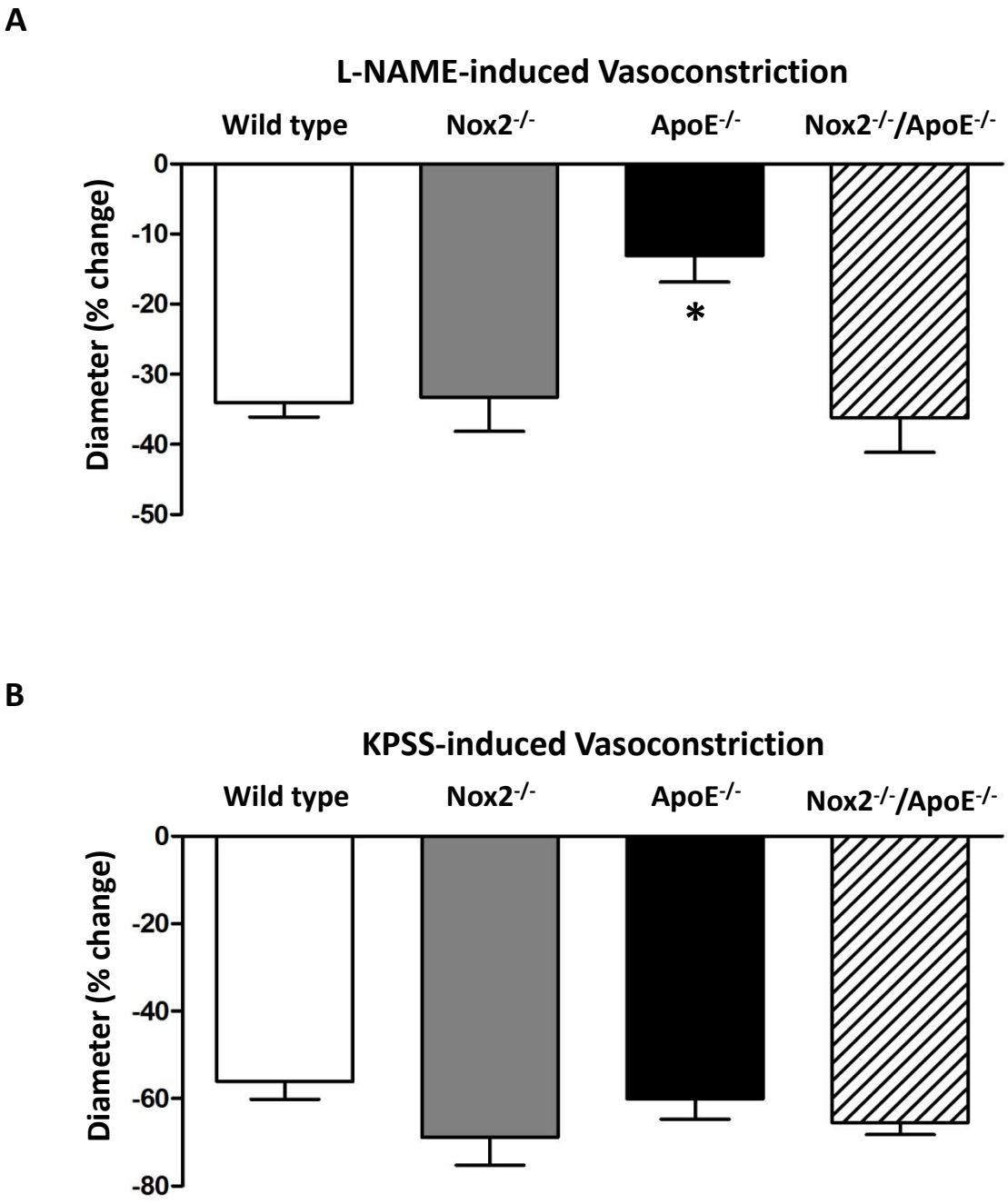


Figure 6. Constrictor responses of isolated middle cerebral arteries (MCA) from wild type, Nox2-deficient ($Nox2^{-/-}$), apolipoprotein E-deficient ($ApoE^{-/-}$) and $Nox2^{-/-}/ApoE^{-/-}$ mice to the NOS inhibitor L-NAME (100 µmol/L) **(A)**. Constrictor responses to high potassium physiological salt solution (KPSS; 122.7 mmol/L) **(B)**. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (**A** and **B**, n=6 for all groups). **P*<0.05 vs. all other groups (one-way ANOVA with a Bonferroni multiple comparison post-hoc test).

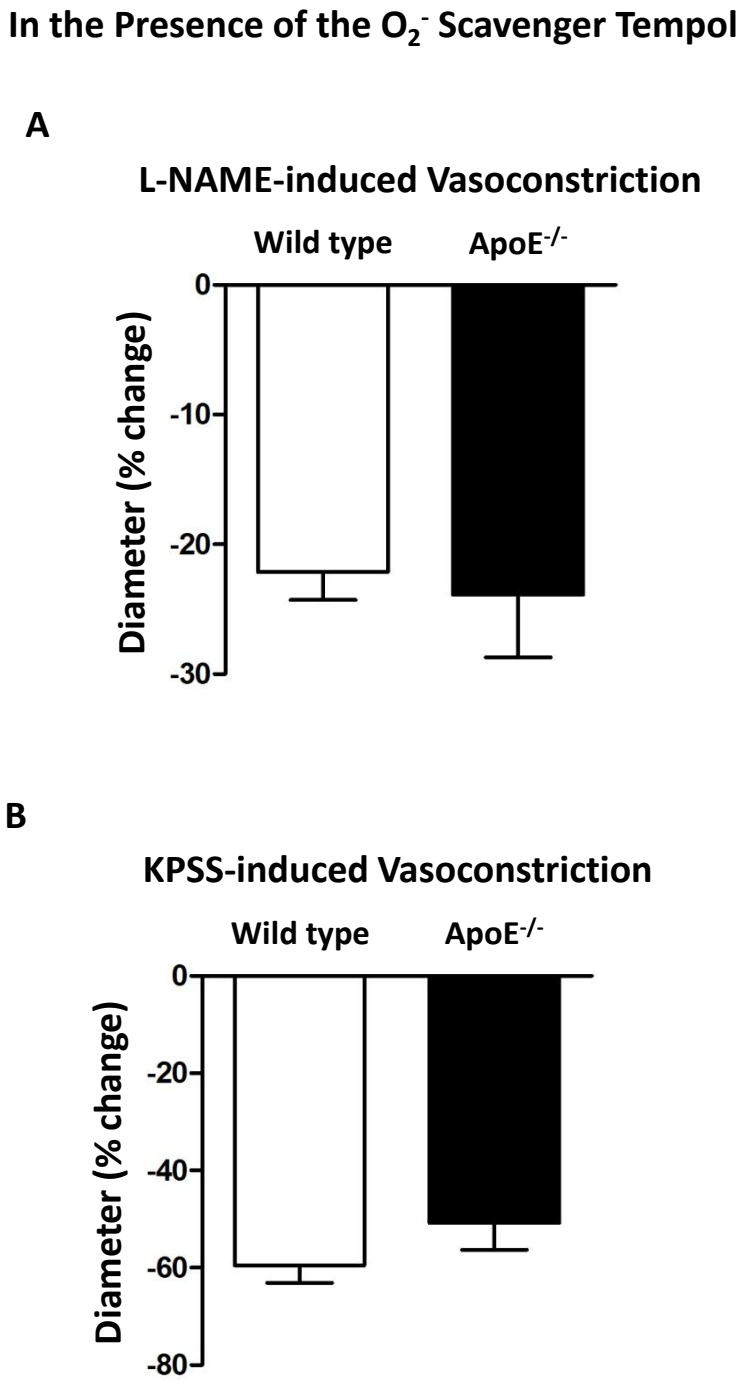


Figure 7. Effect of the O_2^- scavenger tempol (1 mmol/L) on constrictor responses to the NOS inhibitor L-NAME (100 µmol/L) of isolated middle cerebral arteries (MCA) from wild type and apolipoprotein E-deficient ($ApoE^{-/-}$) mice **(A)**. Constrictor responses of isolated MCA to high potassium physiological salt solution (KPSS; 122.7 mmol/L) after treatment with tempol and L-NAME **(B)**. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (**A** and **B**, n=5 for wild type and n=6 for $ApoE^{-/-}$).

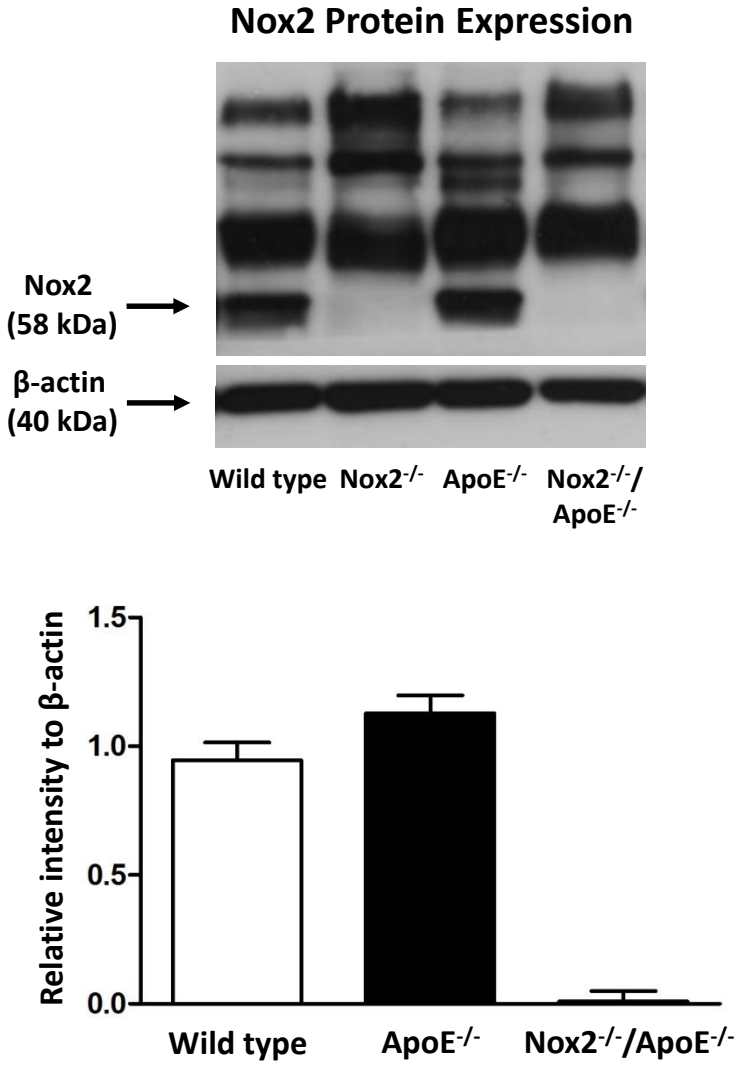


Figure 8. Representative western blot showing protein expression of the NADPH oxidase catalytic subunit, Nox2, in cerebral arteries from wild type, Nox2-deficient ($Nox2^{-/-}$), apolipoprotein E-deficient ($ApoE^{-/-}$) and $Nox2^{-/-}/ApoE^{-/-}$ mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM (n=10 for wild type; n=8 for $ApoE^{-/-}$; n=6 for $Nox2^{-/-}/ApoE^{-/-}$).

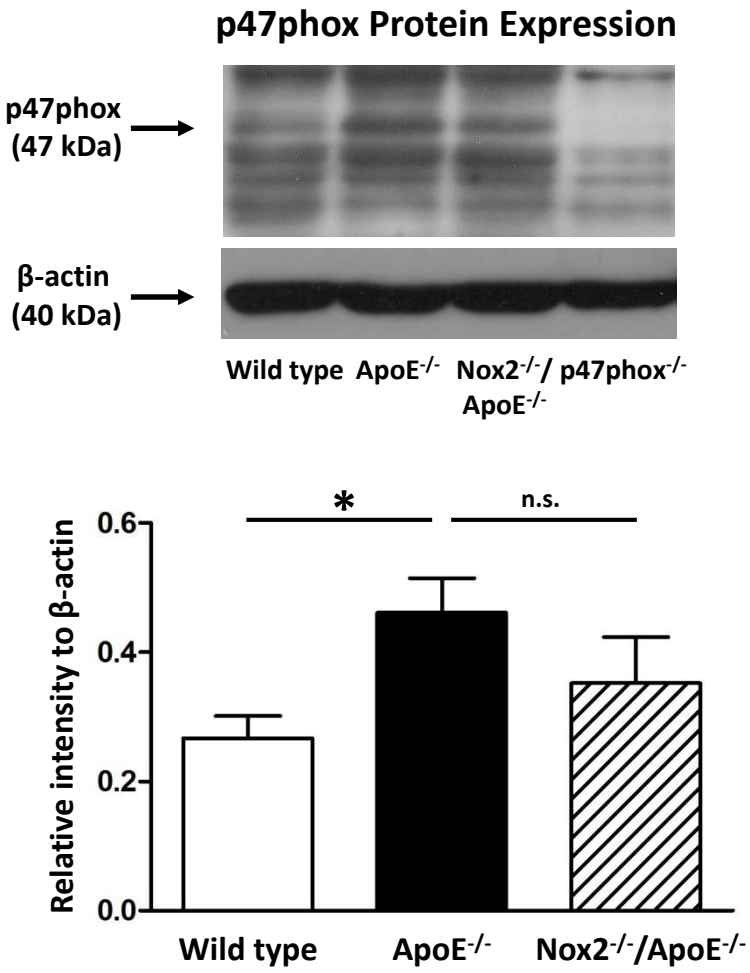
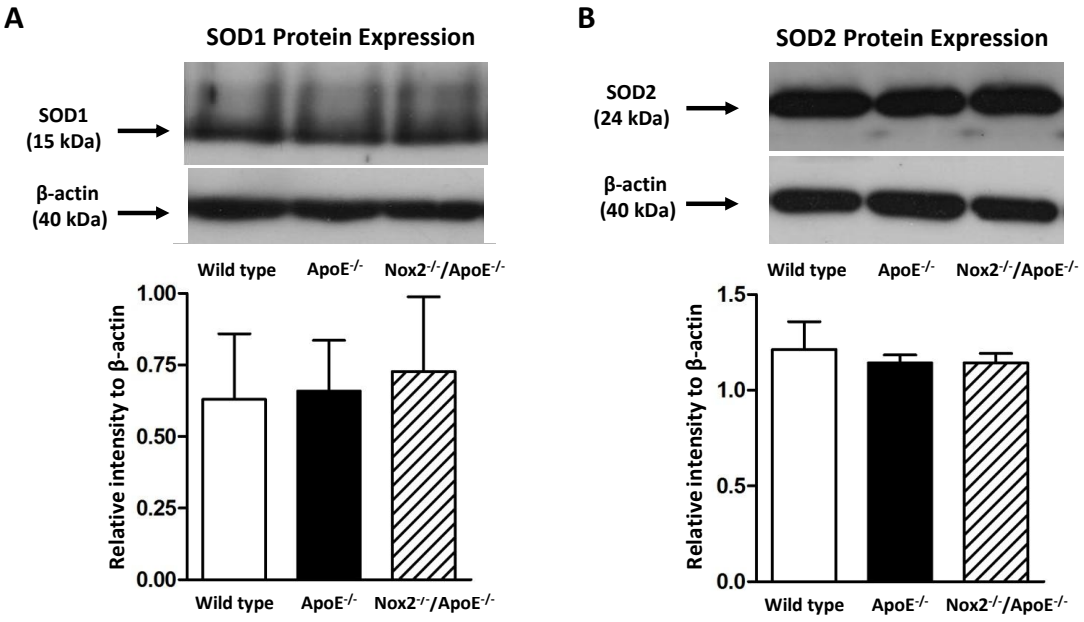


Figure 9. Representative western blot showing protein expression of the Nox2-NADPH oxidase organiser subunit, p47phox, in cerebral arteries from wild type, apolipoprotein E-deficient ($ApoE^{-/-}$), Nox2-deficient ($Nox2^{-/-}$)/$ApoE^{-/-}$ and p47phox-deficient ($p47phox^{-/-}$) mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM (n=7 for all groups). *P<0.05 vs. wild type, n.s. = not significant vs. $Nox2^{-/-}$/$ApoE^{-/-}$ (one-way ANOVA with a Bonferroni multiple comparison post-hoc test).


A
SOD1 Protein Expression
SOD1
(15 kDa)
β-actin
(40 kDa)
Wild type
ApoE-/-
Nox2-/-/ApoE-/-
Relative intensity to β-actin
1.00
0.75
0.50
0.25
0.00
B
SOD2 Protein Expression
SOD2
(24 kDa)
β-actin
(40 kDa)
1.5
1.0
0.5
0.0

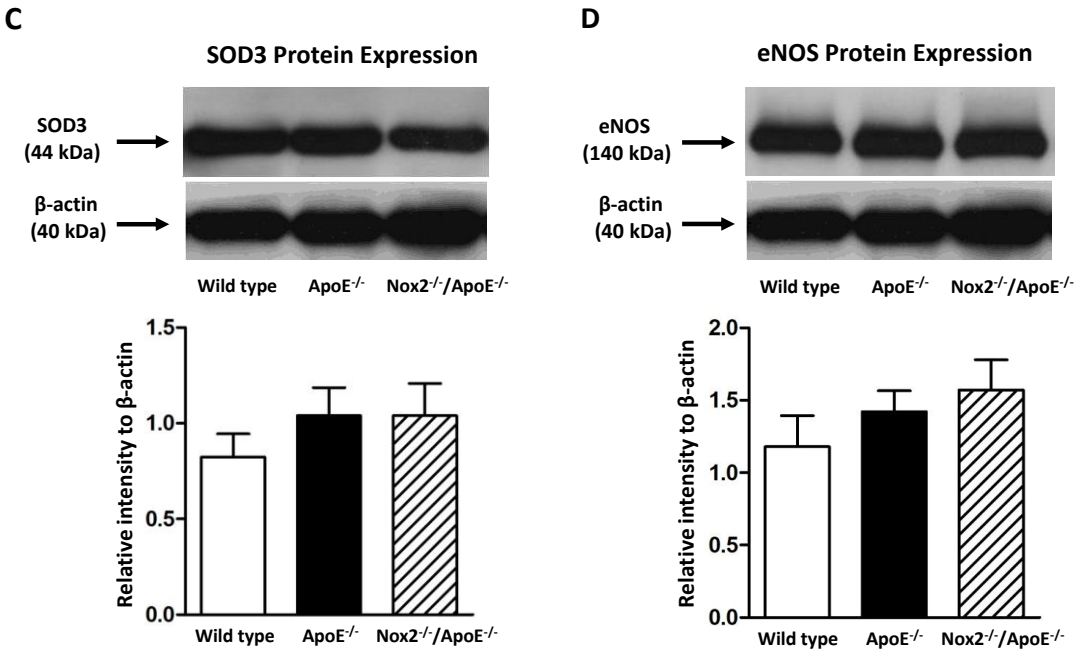

C
SOD3 Protein Expression
SOD3
(44 kDa)
β-actin
(40 kDa)
D
eNOS Protein Expression
eNOS
(140 kDa)
2.0

Figure 10. Representative western blots showing protein expression of superoxide dismutase (SOD) isoforms SOD1 **(A)**, SOD2 **(B)** and SOD3 **(C),** and endothelial nitric oxide synthase (eNOS) **(D)** in cerebral arteries from wild type, apolipoprotein E-deficient ($ApoE^{-/-}$) and Nox2-deficient ($Nox2^{-/-}$)/$ApoE^{-/-}$ mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM (**A**, n=5 for all groups; **B**, n=4 for all groups; **C**, n=3 for all groups; **D**, n=6 for wild type, n=4 for $ApoE^{-/-}$ and n=4 for $Nox2^{-/-}$/$ApoE^{-/-}$).

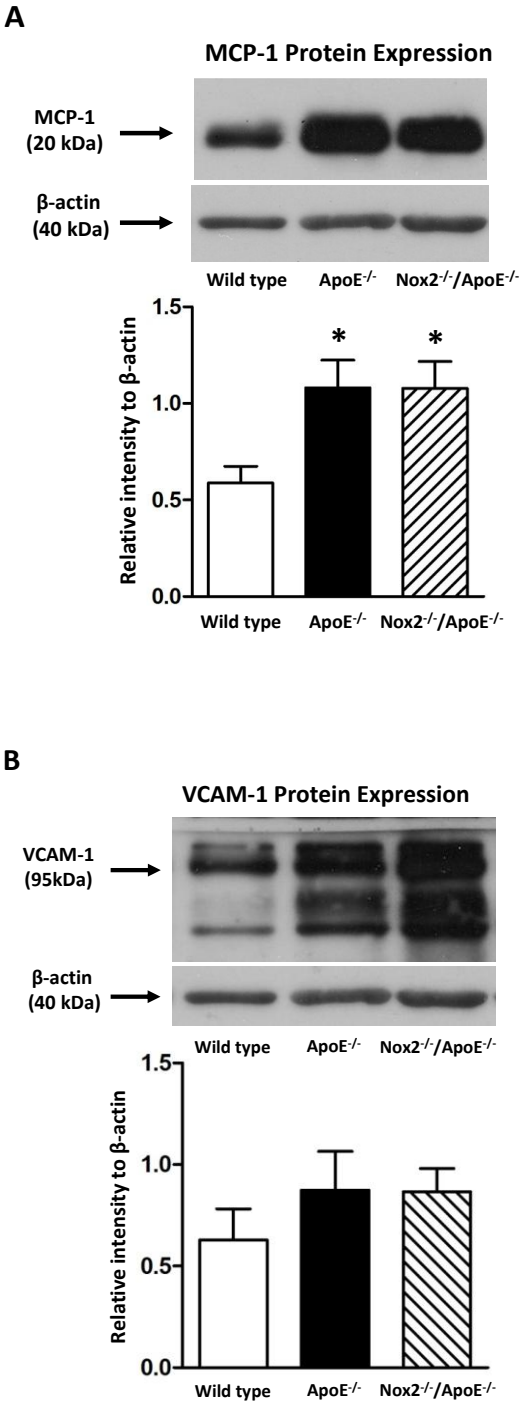


Figure 11. Representative western blots showing protein expression of monocyte chemoattractant protein 1 (MCP-1) **(A)** and vascular cell adhesion molecule 1 (VCAM-1) **(B)** in cerebral arteries from wild type, apolipoprotein E-deficient ($ApoE^{-/-}$) and Nox2-deficient ($Nox2^{-/-}$)/$ApoE^{-/-}$ mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalised to β-actin intensity and given as mean ± SEM (**A & B**, n=6 for all groups). **P*<0.05 vs. wild type (one-way ANOVA with a Bonferroni multiple comparison post-hoc test).

Discussion

The present study provides novel insight into the effects of hypercholesterolaemia on the cerebral circulation. Specifically, the major findings of this study are that, despite the absence of atherosclerotic lesions, cerebral arteries from hypercholesterolaemic mice have: (1) elevated Nox2-dependent O_2^- production; (2) reduced NO·-mediated vasodilator function; and (3) increased expression of the NADPH oxidase organiser subunit, p47phox. Either genetic deletion of Nox2 or acute scavenging of O_2^- with tempol abrogated the decreased NO· function in cerebral arteries during hypercholesterolaemia. Collectively these findings represent the first evidence that augmented production of Nox2-NADPH oxidase-derived O_2^- is a major mediator of cerebral artery dysfunction during hypercholesterolaemia.

Under physiological conditions, reactive oxygen species (ROS) such as O_2^- and its downstream metabolites serve as important cell signalling molecules for the regulation of normal vascular function (Miller *et al.*, 2006a). However, a wealth of experimental evidence indicates that excessive O_2^- production is associated with several diseases affecting the cerebral circulation, such as hypertension and stroke (see Chapter 6, Girouard *et al.*, 2006; Miller *et al.*, 2006b). In Chapter 3, we demonstrated that Nox2-NADPH oxidase contributes to angiotensin II-stimulated O_2^- production by cerebral arteries from male mice. It has been suggested that increased O_2^- production by one or more isoforms of NADPH oxidase may contribute to diminished NO· function in cerebral arterioles of hypercholesterolaemic ApoE$^{-/-}$ mice (Kitayama *et al.*, 2007). However, it is unclear whether O_2^- production is elevated in the cerebral circulation of hypercholesterolaemic mice, and if this O_2^- is derived from Nox2-NADPH oxidase. To test this, we generated a colony of ApoE$^{-/-}$ mice that were also Nox2-deficient (Nox2$^{-/-}$/ApoE$^{-/-}$). In both ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice total plasma cholesterol levels were substantially and similarly elevated compared with wild type, indicating that both genotypes are equally hypercholesterolaemic. Furthermore, there was no difference with respect to either total cholesterol levels or the ratio of HDL:LDL cholesterol between ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ mice, indicating that Nox2 deletion in ApoE$^{-/-}$ mice does not affect the development of hypercholesterolaemia. Using the chemiluminescence probe, L-012, we found that despite the absence of atherosclerotic lesions (see below), basal O_2^-

production by cerebral arteries from hypercholesterolaemic ApoE$^{-/-}$ mice was approximately 2-fold higher than in normocholesterolaemic wild type mice. In genetically related hypercholesterolaemic Nox2$^{-/-}$/ApoE$^{-/-}$ mice, cerebral artery O_2^- production was similar to levels found in arteries from wild type mice. Therefore, these findings indicate that augmented O_2^- production in cerebral arteries from hypercholesterolaemic ApoE$^{-/-}$ mice is dependent on Nox2-NADPH oxidase.

A deficiency in the cofactor tetrahydrobiopterin (BH_4) or its substrate, L-arginine, can lead to eNOS generating large amounts of O_2^-, a phenomenon termed eNOS uncoupling (Vasquez-Vivar *et al.*, 1998; Wever *et al.*, 1997; Xia *et al.*, 1998). Studies of animal models of hypertension and atherosclerosis indicate that uncoupled eNOS may be an important source of O_2^- in the vasculature during disease (Dikalova *et al.*, 2010; Landmesser *et al.*, 2003; Ozaki *et al.*, 2002; Takaya *et al.*, 2007). Furthermore, it has been postulated that NADPH oxidase may be initial source of O_2^- that leads to uncoupling of eNOS (Landmesser *et al.*, 2003). Therefore, it is possible that the augmented O_2^- production in cerebral arteries from hypercholesterolaemic mice may be derived from both Nox2-NADPH oxidase and uncoupled eNOS. To test for evidence of this, we next measured O_2^- production by cerebral arteries from ApoE$^{-/-}$ mice in the presence of the NOS inhibitor L-NAME. We found that L-NAME had no significant effect on O_2^- production by cerebral arteries from ApoE$^{-/-}$ mice, indicating uncoupled eNOS, or any other isoform of NOS, is unlikely to contribute to augmented cerebral artery O_2^- production during hypercholesterolaemia. Thus, our data provide evidence that Nox2-NADPH oxidase is likely to be the sole source of augmented O_2^- production by cerebral arteries during hypercholesterolaemia.

A recent study has found that acetylcholine-induced NO·-dependent dilatation is impaired in cerebral arterioles of ApoE$^{-/-}$ mice (Kitayama *et al.*, 2007; Yamashiro *et al.*, 2010). Similarly, Didion *et al.* (2001) reported that constriction of the basilar artery to the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) is reduced in hypercholesterolaemic monkeys (Didion *et al.*, 2001), which may reflect reduced basal production of NO·, as sGC is a downstream mediator of NO· signalling. Moreover, some (Rossitch *et al.*, 1991; Shimokawa *et al.*, 1988; Stewart-Lee *et al.*, 1991)

but not all (Kitagawa *et al.*, 1994; Simonsen *et al.*, 1991) studies have reported impaired NO·-dependent relaxation in the cerebral circulation of hypercholesterolaemic rabbits. We and others have previously reported that Nox2 is predominantly localised to endothelial cells of mouse cerebral arteries (See Chapter 3, Kazama *et al.*, 2004; Miller *et al.*, 2009). Increased O_2^- production by Nox2 in cells that express NOS isoforms is likely to be of significance for oxidative stress because O_2^- reacts avidly with NO· when both species are generated in the same biological compartment (Thomson *et al.*, 1995). Therefore, we next tested whether augmented Nox2-derived O_2^- leads to decreased cerebral artery NO· function. We assessed basal NO· function in pressurised isolated MCA using L-NAME, whereby the magnitude of the constrictor response was indicative of the degree to which NO· normally attenuates vascular tone. Measuring constrictor responses to L-NAME may be a more physiological representation of endothelial NO· function, as it may better reflect steady-state levels of endothelial-derived NO· in the artery wall than would the response to a strong stimulus, such as acetylcholine, that is not likely to be a relevant endogenous stimulus in the vessel wall. We found that constriction to L-NAME were substantially smaller in MCA from $ApoE^{-/-}$ mice than responses in wild type mice, suggesting that cerebral artery NO· function is diminished during hypercholesterolaemia. We next examined constrictor responses to L-NAME in hypercholesterolaemic $Nox2^{-/-}$/$ApoE^{-/-}$ mice. Consistent with our O_2^- data, L-NAME-induced constriction of MCA from $Nox2^{-/-}$/$ApoE^{-/-}$ mice were comparable to those observed in wild type mice. These findings are in contrast to a recent study of $ApoE^{-/-}$ mice where L-NAME-induced constrictor responses of MCA were similar to those found in wild type mice (Yamashiro *et al.*, 2010). While the reasons for this contradictory finding are unclear, it could be due to differences in the technique used to assess cerebral artery function (perfusion myography in the present study compared with wire myography). Another possible explanation could be that the wild type and $ApoE^{-/-}$ mice studied by Yamashiro *et al.* (2010) were not closely genetically matched as the mice in the present study. This last point notwithstanding, our findings in the present study provide the first direct evidence that Nox2-NADPH oxidase plays a central role in impaired NO· function of cerebral arteries during hypercholesterolaemia.

A previous study reported that impaired acetylcholine-induced NO·-dependent vasodilatation of cerebral arterioles from ApoE$^{-/-}$ mice is restored by the SOD mimetic, tempol (Kitayama *et al.*, 2007). In this study we extended this finding by assessing the effect of tempol on basal NO· levels during hypercholesterolaemia. We found that in the presence of tempol, the magnitude of L-NAME–induced constriction was equivalent in cerebral arteries from wild type and ApoE$^{-/-}$ mice. This acute improvement by tempol of cerebral artery NO· function in ApoE$^{-/-}$ mice suggests that Nox2-NADPH oxidase-derived O_2^- might inactivate NO· during hypercholesterolaemia. It is also conceivable that O_2^- and/or a downstream ROS, such as hydrogen peroxide, might impair NO· signalling by reducing the activity of sGC (Gerassimou *et al.*, 2007; Priviero *et al.*, 2009). However, several studies have reported that responses to NO· donors are normal in cerebral vessels from hypercholesterolaemic animals, including ApoE$^{-/-}$ mice (Didion *et al.*, 2001; Kitagawa *et al.*, 1994; Kitayama *et al.*, 2007; Simonsen *et al.*, 1991; Yamashiro *et al.*, 2010). Thus, smooth muscle signalling in response to NO· is likely to be preserved in cerebral arteries during hypercholesterolaemia and is unlikely to account for the smaller constriction to L-NAME in our ApoE$^{-/-}$ mice. Also, the relatively low level of basal dilatation by NO· in ApoE$^{-/-}$ mice is unlikely to be attributable to reduced rates of NO· generation and/or O_2^- metabolism by SODs, as expression levels of eNOS and all three SOD isoforms were similar between wild type and ApoE$^{-/-}$ mice. To the best of our knowledge, no study has ever examined whether the ApoE gene is expressed and functionally important in cerebral vessels. Therefore, we cannot exclude the possibility that deletion of the ApoE gene, irrespective of the plasma cholesterol level, contributes to the changes we observed in this study.

To explore a possible molecular basis for our findings, we next examined the effect of hypercholesterolaemia on cerebral artery expression of the Nox2. Using western blotting, we observed a Nox2-immunoreactive band at approximately 58 kDa in cerebral artery homogenates from wild type and ApoE$^{-/-}$ mice, which was absent in homogenates from either Nox2$^{-/-}$ or Nox2$^{-/-}$/ApoE$^{-/-}$ mice. Analysis of the intensity of this band, however, revealed no significant difference between wild type and ApoE$^{-/-}$ mice. Thus, hypercholesterolaemia does not appear to influence protein expression levels of the Nox2 catalytic subunit in cerebral arteries. As discussed in Chapter 1, numerous factors regulate

the activity of Nox2 including its association with the cytosolic organiser subunit p47phox. In ApoE$^{-/-}$ mice, cerebral artery expression levels of p47phox protein were approximately 2-fold higher than in wild type mice but were similar to levels found in Nox2$^{-/-}$/ApoE$^{-/-}$ mice. The p47phox subunit plays a critical role in the activation of Nox2-NADPH oxidase. Thus, greater quantities of the p47phox subunit in cerebral arteries during hypercholesterolaemia might result in higher activity of Nox2-NADPH oxidase, and thus may contribute to the augmentation in vascular O_2^- levels.

It is well established that hypercholesterolaemia is a risk factor for the development of atherosclerotic lesions in systemic arteries. In the present study, despite elevated plasma cholesterol levels and in contrast with the aorta, where atherosclerotic lesions were prominent, we found no atherosclerotic lesions in MCA from hypercholesterolaemic ApoE$^{-/-}$ or Nox2$^{-/-}$/ApoE$^{-/-}$mice. Our findings are consistent with a previous study which did not detect any atherosclerotic lesions in pial arterioles of ApoE$^{-/-}$ mice fed a high fat diet for 6-12 months (Kitayama *et al.*, 2007). Furthermore, in other animal models of hypercholesterolaemia/atherosclerosis it has been reported that cerebral arteries do not develop atherosclerotic lesions (Hoekstra *et al.*, 2008; Kitagawa *et al.*, 1994) or develop less severe lesions later in life (Ito *et al.*, 2001). Similarly, in primates, it has been reported that atherosclerotic lesions develop inconsistently, with lesions observed in only approximately half of the animals studied (Didion *et al.*, 2001; Suzuki *et al.*, 2006). Furthermore, studies in humans have revealed that cerebral atherosclerotic lesions develop later in life, and are relatively less severe compared with systemic arteries (D'Armiento *et al.*, 2001; Mathur *et al.*, 1963; Napoli *et al.*, 1999; Sadoshima *et al.*, 1980). Previous studies have shown that intracranial arteries (MCA and basilar artery) have significantly greater activity of the antioxidant enzyme SOD2 compared with extracranial arteries, such as the carotid artery and abdominal aorta (D'Armiento *et al.*, 2001; Napoli *et al.*, 1999). This finding raises the possibility that cerebral arteries have a greater antioxidant capacity, which may contribute to the reduced atherosclerotic lesion formation. Overall, the findings discussed above would suggest that cerebral arteries may be less susceptible to developing atherosclerotic lesions compared with arteries outside of the brain, which may partly be due to better antioxidant protection in cerebral arteries. A second possible explanation for minimal atherogenesis in cerebral arteries may be a

dampening down of the local inflammatory response to hypercholesterolaemia. There is a growing body of evidence to suggest that hypercholesterolaemia induces an inflammatory phenotype within the vasculature (Ishikawa *et al.*, 2004; Stokes *et al.*, 2007a; Stokes *et al.*, 2007b), which is thought to play a pivotal role in the initiation and development of atherogenesis. Furthermore, it has been previously demonstrated that the expression of pro-inflammatory markers, such as the chemokine MCP-1 and the adhesion molecule VCAM-1, are elevated in atherosclerotic lesion prone regions of the aorta, prior to the development of atherosclerotic lesions (Jongstra-Bilen *et al.*, 2006). Therefore, we next sought to determine if protein expression of MCP-1 and VCAM-1 was elevated in cerebral arteries from hypercholesterolaemic ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ compared with wild type mice, as this may reflect a pro-inflammatory state. In the absence of atherosclerotic lesions, we found that protein expression of MCP-1, but not VCAM-1, was elevated in cerebral arteries from hypercholesterolaemic ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ compared with wild type mice. While we have only measured the expression of two pro-inflammatory markers, these findings raise the possibility that the inflammatory response in the cerebral vasculature to hypercholesterolaemia may be less pronounced and that it may occur in parallel with, rather than as a result of, increased Nox2 activity. Clearly, further studies are needed to compare between the cerebral and systemic vasculature with respect to inflammation in response to hypercholesterolaemia and thus to determine if part of the reason for the delayed atherosclerotic lesion formation in cerebral arteries is due to an attenuation of the inflammatory response to hypercholesterolaemia.

Early epidemiological studies reported both positive (Iso *et al.*, 1989; Kannel *et al.*, 1965) and negative (Kagan *et al.*, 1980; Wannamethee *et al.*, 2000) correlations between increased plasma cholesterol levels and stroke incidence. Consequently, opinions have been divided as to whether elevated plasma cholesterol is a risk factor for stroke (Demchuk *et al.*, 1999; Landau, 1999). It has been suggested that the aforementioned conflicting reports may be due to earlier studies only measuring total cholesterol rather than separately measuring HDL and LDL cholesterol levels. Indeed, in a long term prospective study, when LDL and total cholesterol was assessed separately, a positive and significant association was found between plasma LDL cholesterol levels and stroke

incidence (Imamura *et al.*, 2009). Furthermore, a clinical trial demonstrated that stroke patients who received statin therapy had reduced LDL cholesterol levels and importantly reduced risk of secondary stroke (Amarenco *et al.*, 2006). Thus, recent clinical data would suggest that elevated LDL cholesterol is indeed a risk factor for stroke. A second possible explanation for the conflicting clinical data discussed above could be the fact that early reports classified stroke as a single disease rather than separating it into different stroke subtypes. As stroke is a heterogeneous disease of different etiological origins, elevated cholesterol levels may be important for some stroke subtypes but not others. Indeed, Imamura *et al.* (2009) showed that increasing LDL cholesterol is associated with significantly more atherothrombotic and lacunar, but not haemorrhagic strokes (Imamura *et al.*, 2009). Taken together with the findings in the present study showing that hypercholesterolaemia has profound effects on cerebral artery function, these recent clinical studies suggest that hypercholesterolaemia may be a risk factor for at least some stroke subtypes.

CHAPTER 6

EFFECT OF TRANSIENT CEREBRAL ISCHAEMIA ON MIDDLE CEREBRAL ARTERY FUNCTION: ROLE OF NOX2-NADPH OXIDASE

Introduction

As discussed throughout this thesis, a growing body of evidence to suggests that reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), play crucial roles in the modulation of vascular function during both health and in the pathogenesis of several diseases. Under physiological conditions, ROS have been shown to mediate cerebral artery responses to a number of stimuli, including angiotensin II and arachidonic acid (see Chapter 4, Miller *et al.*, 2005; Modrick *et al.*, 2009). However, evidence suggests that elevated ROS levels may play an important role in cerebral vascular dysfunction associated with a number of diseases affecting the cerebral circulation, such as hypertension and hypercholesterolaemia (see Chapter 5, Girouard *et al.*, 2006; Kazama *et al.*, 2003). O_2^- and nitric oxide (NO·) react avidly when present in the same biological compartment (Thomson *et al.*, 1995). As such, one of the major consequences of elevated ROS production would be a reduction in the bioavailability of NO·. A reduction in the bioavailability of NO· has numerous effects on the vasculature, including impaired endothelium-dependent vasorelaxation (see Chapter 5, Capone *et al.*, 2010; Chrissobolis *et al.*, 2010; Didion *et al.*, 2003; Kazama *et al.*, 2004; Kitayama *et al.*, 2007), which is a classic hallmark of endothelial dysfunction.

The brain has minimal energy reserves and is therefore dependent on a continuous supply of oxygen and nutrients in order to maintain function. Ischaemic stroke is a devastating and prevalent disease and that occurs as a result of a severe reduction in blood flow to the brain. The middle cerebral artery (MCA) territory is the most commonly occluded region with MCA stroke accounting for between 15 % and 30 % of strokes (Mohr *et al.*, 2004). Previous studies have identified numerous mediators that may contribute to neuronal cell death and subsequent brain infarct development following ischaemic stroke, such as ROS, immune cells and inflammatory cytokines (for example: Brait *et al.*, 2010; Iadecola *et al.*, 2001a; Kahles *et al.*, 2007; McCann *et al.*, 2008; Yilmaz *et al.*, 2006). However, much less in known about cerebral artery function following cerebral ischaemia.

Following cerebral ischaemia, the function and integrity of cerebral blood vessels are critical to support cerebral blood flow and thus minimise further neuronal injury and neurological impairment. Occlusion of cerebral arteries and subsequent reperfusion causes cerebral artery damage after as little as 10 min of ischaemia and cerebral artery function progressively worsens (Cipolla *et al.*, 2002; Mayhan *et al.*, 1988; Rosenblum *et al.*, 1997). It has been suggested that elevated vascular O_2^- production following reperfusion may be a cause of endothelial dysfunction and this may result in further neuronal damage (Fagan *et al.*, 2004). Indeed, excessive ROS production (Miller *et al.*, 2006; Mori *et al.*, 1999) and impaired NO· function has been reported in experimental models of cerebral ischaemia-reperfusion (Cipolla *et al.*, 1997; Mayhan *et al.*, 1988). Nox2-NADPH oxidases may be an important source of ROS within cerebral arteries under physiological conditions (Miller *et al.*, 2009) and excessive production of ROS by Nox2-NADPH oxidase has been implicated in the development of endothelial dysfunction associated with other diseases affecting the cerebral circulation such as hypertension and hypercholesterolaemia (see Chapter 5, Girouard *et al.*, 2006; Kazama *et al.*, 2004). These studies suggest that Nox2-NADPH oxidase may be an important source of pathological levels of ROS. However, no study has definitively tested the role of Nox2-NADPH oxidase in excessive ROS production and impaired NO· function following cerebral ischaemia-reperfusion Therefore, in the present study we firstly tested whether cerebral artery O_2^- production is augmented 24 h after transient cerebral ischaemia-reperfusion in mice and whether this is associated with impaired NO· function. Secondly, using Nox2-deficient ($Nox2^{-/-}$) mice, we tested whether any such changes in O_2^- production and NO· function were dependent on Nox2-NADPH oxidase.

Materials and Methods

All procedures were approved by the institutional animal ethics committee. In total, 35 male C57Bl6/J wild type (8-12 weeks of age; 26.0 ± 0.3 g) and 12 male Nox2-deficient ($Nox2^{-/-}$) mice (8-12 weeks of age; 27.5 ± 0.6 g) were studied. All mice were bred at Monash University and housed in an approved animal holding facility.

Cerebral Ischaemia

Middle Cerebral Artery Occlusion Surgical Procedure

Focal cerebral ischaemia was induced in mice by transient intraluminal filament-induced MCA occlusion (MCAO). Mice were anaesthetised with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Rectal temperature was maintained at 37.5°C ± 0.5°C with a heat lamp (100W PT-2046, Exo Terra, Australian Reptiles Pty. Ltd., Australia) controlled by an temperature regulator (Digitemp EX1310TC, Extech Equipment Pty. Ltd., Australia) throughout the procedure and until animals regained consciousness. Regional cerebral blood flow (rCBF) in the area of the cortex supplied by the MCA (approximately 2 mm posterior and 5 mm lateral to bregma) was monitored and recorded prior to the induction of cerebral ischaemia and for up to 30 min after the beginning of reperfusion using trans-cranial laser-Doppler flowmetry (PF5010 LDPM Unit, Perimed, Sweden). Under a dissecting microscope (MZ6, Leica Microsystems, Germany), the right external carotid artery (ECA) was carefully cleared of connective tissue. A branch of the ECA was cauterised and the ECA was ligated and cut, forming an ECA stump. The internal carotid artery (ICA) and common carotid artery (CCA) was cleared of connective tissue and carefully separated from the adjacent vagus nerve. The proximal CCA was clamped and tension was applied to the ICA with a suture bridge. A small cut was made in the ECA stump and a 6-0 nylon monofilament with a silicone coated tip (diameter: 0.21 mm; Doccol Co., Redlands, California, USA) was inserted and advanced approximately 11 mm distal to the carotid bifurcation along the ICA until it occluded the MCA at its origin at the Circle of Willis. Severe (approximately 75 %) reduction in rCBF in the territory supplied by the MCA, measured using real time trans-cranial laser-Doppler flowmetry, confirmed correct placement of the filament. The filament was held in place by tethering it to the ECA stump and the artery clamp was then removed from the CCA and the suture bridge

was released. Occlusion of the MCA was maintained for 30 min and the filament was then retracted allowing reperfusion (23.5 h), which was confirmed by laser-Doppler flowmetry. The wound was then closed, covered with betadine and spray dressing, and the animal was allowed to recover.

Some mice were subjected to sham MCAO surgery. This involved exposure of the right CCA and ECA, but not filament insertion, for the duration of the typical ischaemic period (i.e. 30 min). Following this period, the wound was closed, covered with betadine and spray dressing, and the animal was allowed to recover.

Evaluation of Neurological Function

At the end of the reperfusion period, neurological assessment was performed using a modified version of a 5 point scoring system (Bederson *et al.*, 1986; Iadecola *et al.*, 2001b) and a wire-hanging test (Brait *et al.*, 2010; Hattori *et al.*, 2000). Both of these techniques have been used extensively to assess neurological impairment in mice subjected to cerebral ischaemia (Cho *et al.*, 2005; Iadecola *et al.*, 2001b; Jackman *et al.*, 2009). Where possible, neurological assessment was performed by an investigator blinded to the experimental treatment.

Five Point Scoring System

Mice were assigned a score from 0 to 4 according to the following criteria: 0, normal motor function; 1, flexion of torso and forelimb exclusively to either side when mouse is lifted by the tail; 2, circling in either direction when held by the tail on a flat surface, but normal posture at rest; 3, leaning to either side at rest; 4, no spontaneous motor function (mice rolling uncontrollably in their cage were also assigned a score of 4). The tests were carried out sequentially and if a mouse exhibited the appropriate behaviour at one step but not the following step, it was assigned the score of the former step. However, this was not the case when assigning a score of 4, as this score indicates that the mice were unable to move, and therefore were unable to exhibit the appropriate behaviour for scores 1 or 2.

Wire-hanging test

Mice were allowed to grip the middle of a wire (approximately 1 mm diameter), suspended horizontally between two poles 60 cm apart, elevated 36 cm above a padded surface. Time suspended from the wire was recorded in seconds, with a maximum time of 60 sec. Each mouse performed the test three times, at five min intervals.

Evaluation of Cerebral Infarct and Oedema Volume

Mice were euthanased at 24 h by overdose of isoflurane, followed by decapitation. The brain was rapidly removed and frozen over liquid nitrogen. Brains were sectioned (30 µm coronal sections; 420 µm apart) using a Leica CM1850 cryostat and thaw mounted onto 0.1 % poly-L-lysine coated slides. Sections were allowed to air dry for 30 min and then immersed in 0.1 % thionin (2 min), rinsed with distilled H_2O, immersed in 70 % ethanol (EtOH; 2 min) followed by 100 % EtOH (2 min). Slides were dried, dipped in xylene and cover slipped with DPX mounting media. Stained sections were imaged with a CCD camera (Cohu Inc., San Diego, California, USA) mounted above a light box (Biotec-Fischer Colour Control 5000, Reiskirchin, Germany). Total infarct volume was quantified using ImageJ image analysis software (Version 1.42q, NIH), correcting for brain oedema, according to the following formula: *CIV = [RIA – (RHA – LHA)] × thickness of slice* (CIV, corrected infarct volume; RIA, right hemisphere infarct area; RHA, right hemisphere area; LHA, left hemisphere area). Oedema volume was estimated according to the formula: *OV = (RHA – LHA) × thickness of slice* (OV, Oedema volume). Infarct and oedema volumes for all sections were totalled and expressed as mm^3.

Quantification of O_2^- Production by Middle Cerebral Arteries Following Cerebral Ischaemia

L-012-enhanced Chemiluminescence

MCA were isolated from the contralateral (non-ischaemic) and ipsilateral (ischaemic) cerebral hemispheres following MCAO. MCA were isolated from the left and right cerebral hemispheres after sham surgery (anatomically corresponding to non-ischaemic and ischaemic MCA). Basal O_2^- production by non-ischaemic/left and ischaemic/right MCA from male wild type (following MCAO or sham surgery) and $Nox2^{-/-}$ (following MCAO

surgery) mice was measured by 100 µmol/L L-012-enhance chemiluminescence as described in Chapter 2. Following the measurement of basal O_2^- production, 10 µmol/L of phorbol-12, 13-dibutyrate (PDB; activator of Nox2-NADPH oxidase) was added to each well and O_2^- production was measured for a further 20 min. Basal counts were subtracted from counts in the presence of PDB from the same MCA and O_2^- production was normalized to dry tissue weight.

Vascular Reactivity Studies

Non-ischaemic and ischaemic MCA from male wild type and Nox2$^{-/-}$ mice following MCAO were mounted between two microcannulae in a pressure myograph (Living Systems Instrumentation Inc.) as described in Chapter 2. Following an equilibration period, MCA were exposed to high potassium physiological salt solution (KPSS; 122.7 mmol/L K^+) to test viability. After a washout and recovery period, experimental protocols were performed as described below. In all experiments, intraluminal diameter was tracked by a video dimension analyser and constrictor responses were expressed as percent change in intraluminal diameter.

Constrictor responses to L-NAME

A single concentration of the NO· synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L; for approximately 30 min) was added extraluminally to non-ischaemic and ischaemic MCA from male wild type or Nox2$^{-/-}$ mice after MCAO surgery.

Constrictor responses to phenylephrine

Cumulative concentrations of the α-adrenoceptor agonist phenylephrine (1 nmol/L – 1 µmol/L; ≥ 5 min between additions) were added extraluminally to non-ischaemic and ischaemic MCA from male wild type mice after MCAO surgery.

Constrictor responses to U46619

Cumulative concentrations of the thromboxane A_2 mimetic U46619 (1 nmol/L – 1 µmol/L; ≥ 10 min between additions) were added extraluminally to non-ischaemic and ischaemic MCA from male wild type mice after MCAO surgery.

Drugs

DPX mounting media was purchased from BDH Chemicals Ltd. (United Kingdom), ketamine from Parnell Laboratories (Australia), L-012 from Wako Pure Chemicals (Japan) and PDB from Calbiochem (USA). U46619 (in methyl acetate) was purchased from Sapphire Biosciences (Australia), xylazine from Troy laboratories (Australia), xylene from Scharlau Chemie (Spain) and all other drugs from Sigma. L-012 was prepared at 100 mmol/L in 100 % DMSO and subsequently diluted in Krebs-HEPES. PDB was prepared and 10 mmol/L in 100 % DMSO and subsequently diluted in Krebs-HEPES. U46619 was firstly prepared at 1 mmol/L in 100 % EtOH and subsequently diluted in Krebs-bicarbonate. All other drugs for myograph experiments were dissolved and diluted in Krebs-bicarbonate. For experiments using L-012 and PDB, the final concentration of DMSO was ≤0.2 %.

Data Analysis

All results (except for neurological scores) are presented as mean ± SEM. Statistical comparisons were performed using one or two-way ANOVA with a Bonferroni multiple comparison post hoc test, Mann-Whitney test or paired/unpaired *t* test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Outcome Following Cerebral Ischaemia

Regional Cerebral Blood Flow

Upon insertion of the filament, regional cerebral blood flow (rCBF; expressed as a percentage of pre-ischaemic levels, i.e. prior to the occlusion of the MCA with the filament) was similarly reduced by approximately 75 % relative to pre-ischaemic levels in wild type and Nox2$^{-/-}$ mice (Figure 1A). Following 30 min of ischaemia, the filament was withdrawn and rCBF initially returned to approximately 100 % and then gradually reduced to approximately 60 % of pre-ischaemic levels after 30 min of reperfusion in both wild type and Nox2$^{-/-}$ mice (Figure 1A). In wild type mice that underwent sham surgery, rCBF remained at approximately 100 % for the duration of the monitoring period.

Neurological Outcome

Twenty-four hours after the induction of ischaemia, the mean neurological score was significantly lower in Nox2$^{-/-}$ mice compared with wild type mice (Figure 1B; P<0.05). Furthermore, hanging wire times were significantly longer in Nox2$^{-/-}$ compared with wild type mice (Figure 1C; Nox2$^{-/-}$, 37.0 ± 6.7 s vs. wild type, 19.8 ± 4.9 s, P<0.05). In wild type mice that underwent sham surgery, all except for 1 animal received a neurological score of 0 with the remaining 1 receiving a score of 1. Furthermore, time suspended on the wire was 57.3 ± 2.8 s (n=7), indicating that these mice had normal neurological function.

Cerebral Infarct and Oedema Volume

Following ischaemia-reperfusion, Nox2$^{-/-}$ mice had significantly smaller infarcts compared with wild type mice (Figure 2A, C & D; Nox2$^{-/-}$, 14.7 ± 3.8 mm^3 vs. wild type, 36.3 ± 3.4 mm^3, P<0.05). As expected, there was no evidence of infarcts in brains from wild type mice following sham surgery. There was a trend for oedema volume to be smaller in Nox2$^{-/-}$ compared with wild type mice, however, this failed to reach statistical significance (Figure 2B; Nox2$^{-/-}$, 8.3 ± 4.2 mm^3 vs. wild type, 11.8 ± 2.3 mm^3, P>0.05).

O_2^- Production by Middle Cerebral Arteries from Wild Type and Nox2-deficient Mice Following Cerebral Ischaemia

Following MCAO, basal O_2^- production was approximately 2-fold greater in ischaemic MCA from wild type mice compared with non-ischaemic MCA (Figure 3A; $P<0.05$) and levels were comparable to levels generated by non-ischaemic left and right MCA following sham surgery(left MCA, 33.7 ± 11.2; right MCA, 36.7 ± 11.2; non-ischaemic MCA, 23.7 ± 3.3, 10^3 counts/mg of dry tissue weight, n=7 for left and right MCA and n=9 for non-ischaemic MCA). In wild type sham animals, there was no difference in basal O_2^- production between right (anatomically corresponding to ischaemic MCA) and left (anatomically corresponding to non-ischaemic MCA) MCA (Figure 3B). The Nox2-NADPH oxidase activator PDB increased O_2^- production above basal levels in all arteries (e.g. ischaemic MCA, basal 51.7 ± 12.5 vs. PDB 2783.6 ± 689.4, 10^3 counts/mg of dry tissue weight, n=9 for both groups). However, PDB-stimulated O_2^- production by ischaemic MCA was approximately 6-fold higher compared with non-ischaemic MCA (Figure 4A; $P<0.05$). In contrast, and consistent with basal O_2^- production, PDB-stimulated O_2^- production was similar between right and left MCA from sham mice (Figure 4B) and levels were comparable to levels generated by non-ischaemic MCA (left MCA, 641.5 ± 32.6; right MCA, 556.8 ± 51.5; non-ischaemic MCA, 533.9 ± 73.0, 10^3 counts/mg of dry tissue weight, n=7 for left and right MCA and n=9 for non-ischaemic MCA).

In contrast to wild type mice following MCAO, basal O_2^- production was similar between non-ischaemic and ischaemic MCA from $Nox2^{-/-}$ mice (Figure 5A). Furthermore, PDB had no significant effect on O_2^- production by either non-ischaemic or ischaemic MCA from $Nox2^{-/-}$ mice (e.g. Ischaemic MCA, basal 19.7 ± 2.9 vs. PDB 17.9 ± 7.0, 10^3 counts/mg of dry tissue weight, n=6 for both groups). PDB-stimulated O_2^- production was similar between non-ischaemic and ischaemic MCA from $Nox2^{-/-}$ mice (Figure 5B).

NO· Function in Middle Cerebral Arteries from Wild Type and Nox2-deficient Mice Following Cerebral Ischaemia

During the equilibration period vessels from wild type mice did not consistently develop significant myogenic tone. Furthermore, baseline diameters of non-ischaemic and ischaemic MCA from wild type mice were similar (Table 1). The magnitude of L-NAME-

induced constriction (measured from baseline diameter) of ischaemic MCA from wild type mice was less than 50 % of that in non-ischaemic MCA (Figure 6A, $P<0.05$). Furthermore, constrictor responses to phenylephrine were significantly augmented in ischaemic MCA from wild type mice compared with non-ischaemic arteries (Figure 6B, $P<0.05$). For example, at 1 µmol/L, phenylephrine constricted ischaemic MCA from wild type mice by approximately 17 % compared with approximately 8 % in non-ischaemic MCA. In contrast, vasoconstrictor responses to U46619 were similar between non-ischaemic and ischaemic MCA from wild type mice (Figure 7).

During the equilibration period, vessels from $Nox2^{-/-}$ mice did not consistently develop significant myogenic tone. Furthermore, baseline diameters of non-ischaemic and ischaemic MCA from $Nox2^{-/-}$ mice were similar (Table 1). The magnitude of L-NAME-induced vasoconstriction (measured from baseline diameter) was similar between non-ischaemic and ischaemic MCA from $Nox2^{-/-}$ mice (Figure 8).

	Wild Type		**$Nox2^{-/-}$**	
	Non-ischaemic	**Ischaemic**	**Non-ischaemic**	**Ischaemic**
Diameter (µm)	118 ± 3 (19)	122 ± 2 (18)	114 ± 5 (6)	111 ± 8 (6)

Table 1. Intraluminal diameters of isolated non-ischaemic and ischaemic middle cerebral arteries from wild type and $Nox2^{-/-}$ mice (n numbers given in brackets).

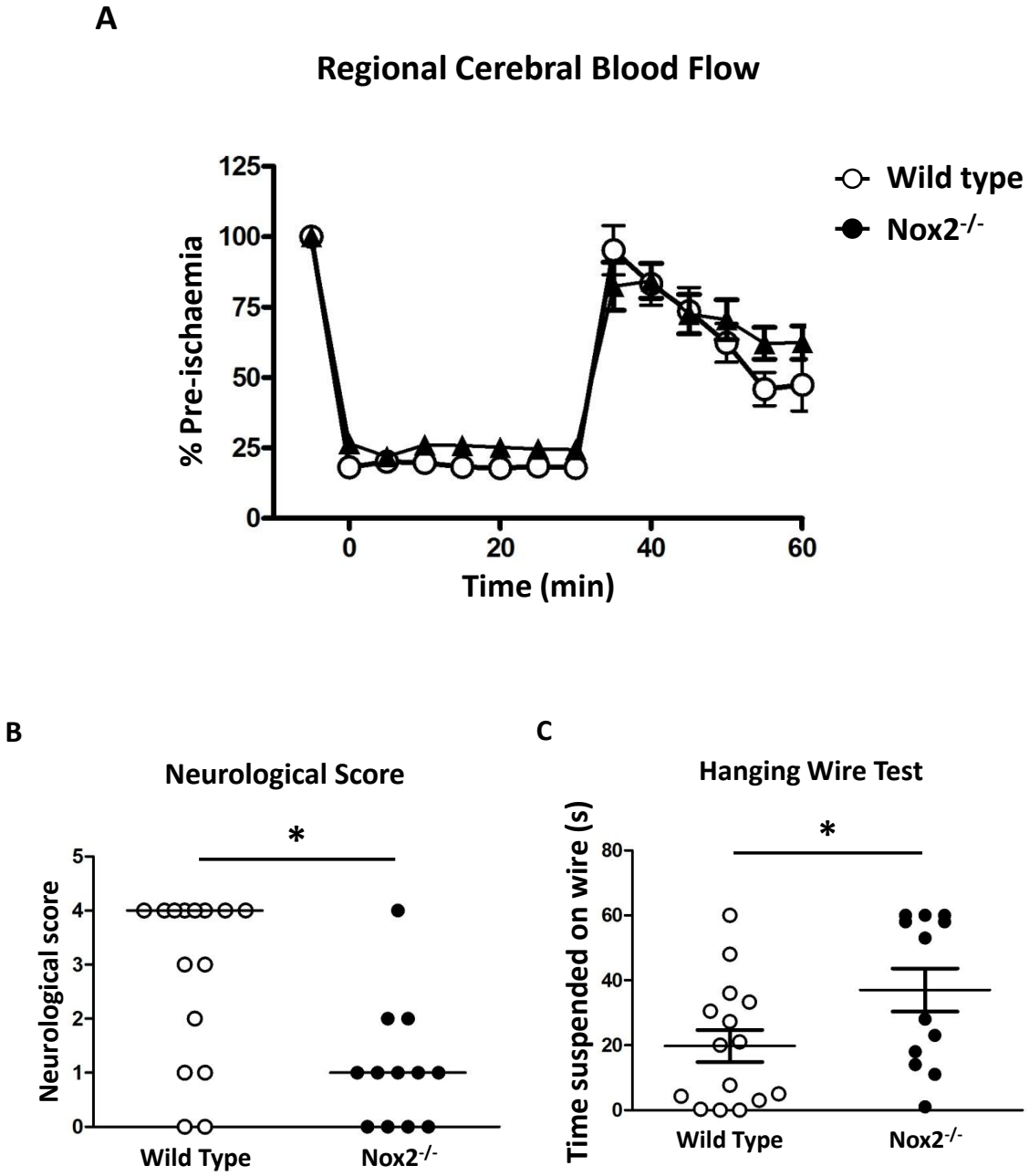


Figure 1. Regional cerebral blood flow **(A)**, neurological score **(B)** and hanging wire test **(C)** in wild type and Nox2-deficient ($Nox2^{-/-}$) mice. Regional cerebral blood flow was measured during and after 30 min cerebral ischaemia. Neurological score and hanging wire test was performed 24 h after cerebral ischaemia-reperfusion. **(A & C)** Results are presented as mean ± SEM; **(B)** line represents median value (**A**, n=15 for wild type and n=12 for $Nox2^{-/-}$; **B & C**, n=15 for wild type n=12 for $Nox2^{-/-}$). **(B)** *P<0.05 vs. $Nox2^{-/-}$ (Mann-Whitney test); **(C)** *P<0.05 vs. $Nox2^{-/-}$ (unpaired *t* test).

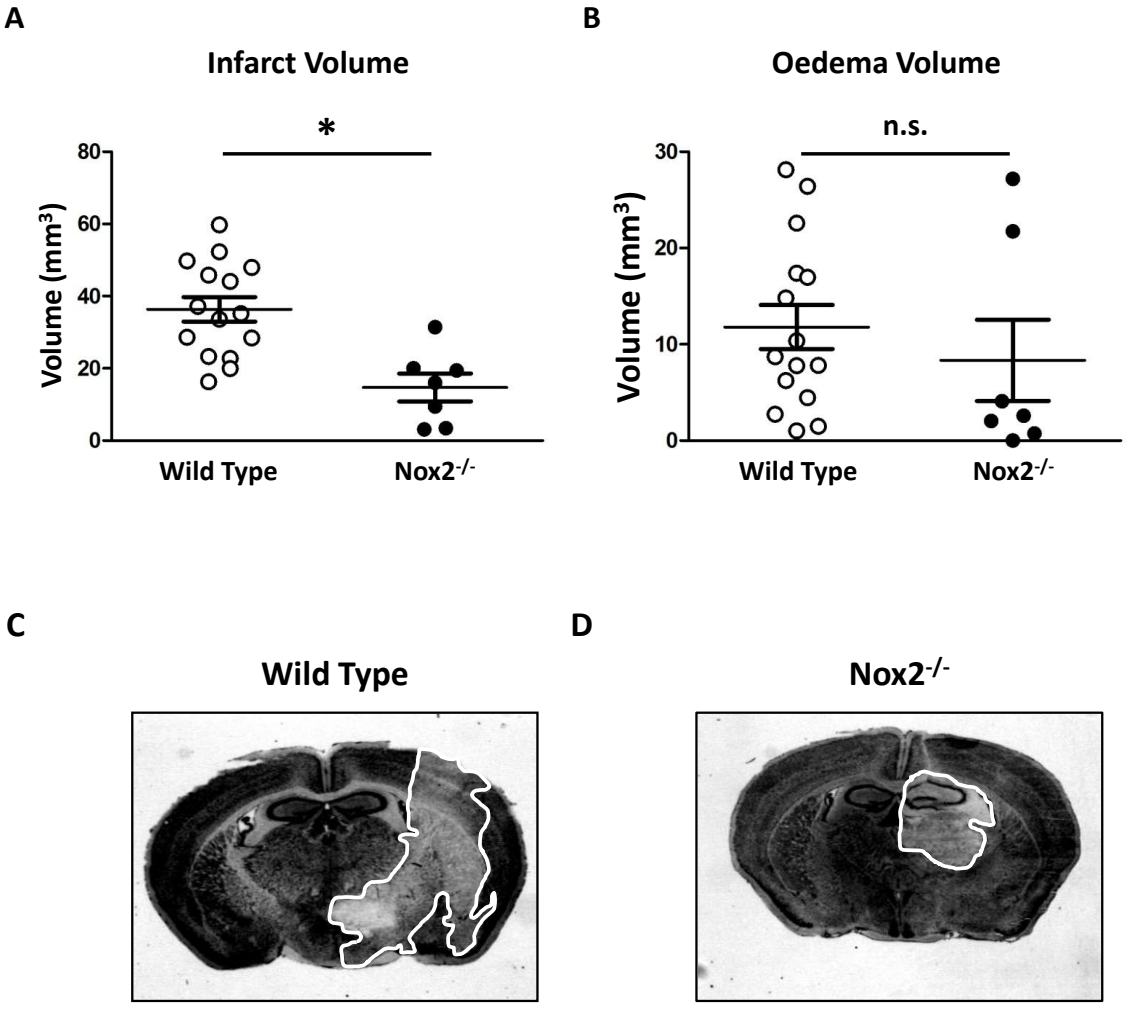


Figure 2. Cerebral infarct **(A)** and oedema **(B)** volumes measured in wild type and Nox2-deficient ($Nox2^{-/-}$) mice 24 h after cerebral ischaemia-reperfusion. Representative coronal brain sections from wild type **(C)** and $Nox2^{-/-}$ **(D)** mice 24 h after cerebral ischaemia-reperfusion with the infarct area outlined in white. All results are presented as mean ± SEM (**A & B**, n=15 for wild type and n=7 for $Nox2^{-/-}$; **C**, representative of n=15; **D**, representative of n=7). **(A)** **P*<0.05 vs. wild type (unpaired *t* test), **(B)** n.s. = not significant.

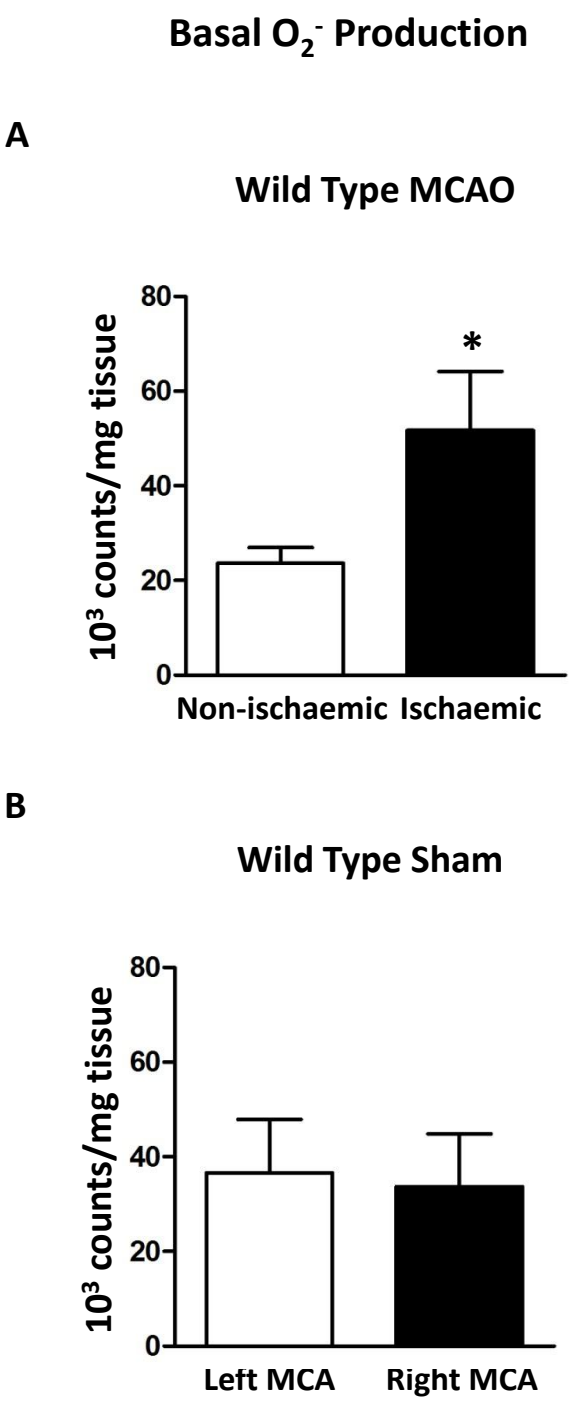


Figure 3. Basal O_2^- production by non-ischaemic and ischaemic middle cerebral arteries (MCA) from wild type mice 24 h after cerebral ischaemia-reperfusion **(A)**. Basal O_2^- production by MCA from the left and right cerebral hemispheres (anatomically corresponding to the non-ischaemic and ischaemic cerebral hemispheres, respectively) from wild type mice 24 h after sham surgery **(B)**. O_2^- production was measured using 100 µmol/L L-012-enhanced chemiluminescence **(A & B)**. All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (**A**, n=9 for both groups; **B**, n=7 for both groups). ***P***<0.05 vs. non-ischaemic MCA (paired *t* test).

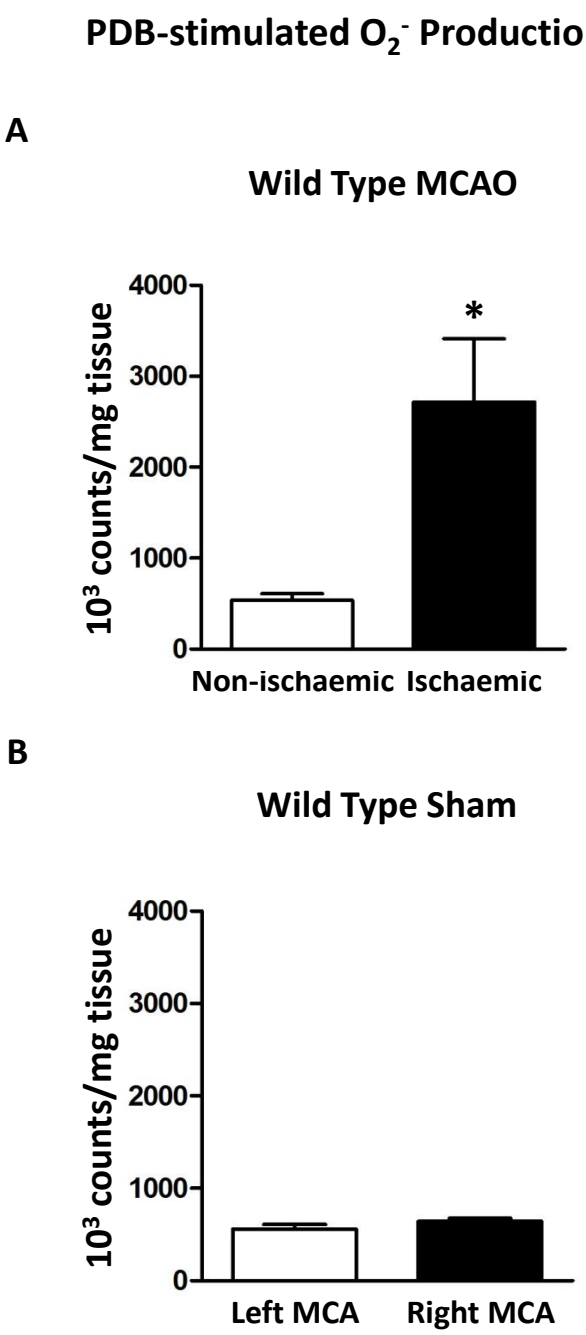


Figure 4. Phorbol-12, 13 dibutyrate (PDB; 10 µmol/L) -stimulated O_2^- production by non-ischaemic and ischaemic middle cerebral arteries (MCA) from wild type mice 24 h after cerebral ischaemia-reperfusion **(A)**. PDB (10 µmol/L)-stimulated O_2^- production by MCA from left and right cerebral hemispheres (anatomically corresponding to the non-ischaemic and ischaemic cerebral hemispheres, respectively) from wild type mice 24 h after sham surgery **(B)**. O_2^- production was measured using 100 µmol/L L-012-enhanced chemiluminescence **(A & B)**. PDB-stimulated O_2^- production represents counts in PDB-treated arteries minus basal counts (i.e. in the absence of PDB). All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (**A**, n=9 for both groups; **B**, n=7 for both groups). ***P***<0.05 vs. non-ischaemic MCA (paired *t* test).

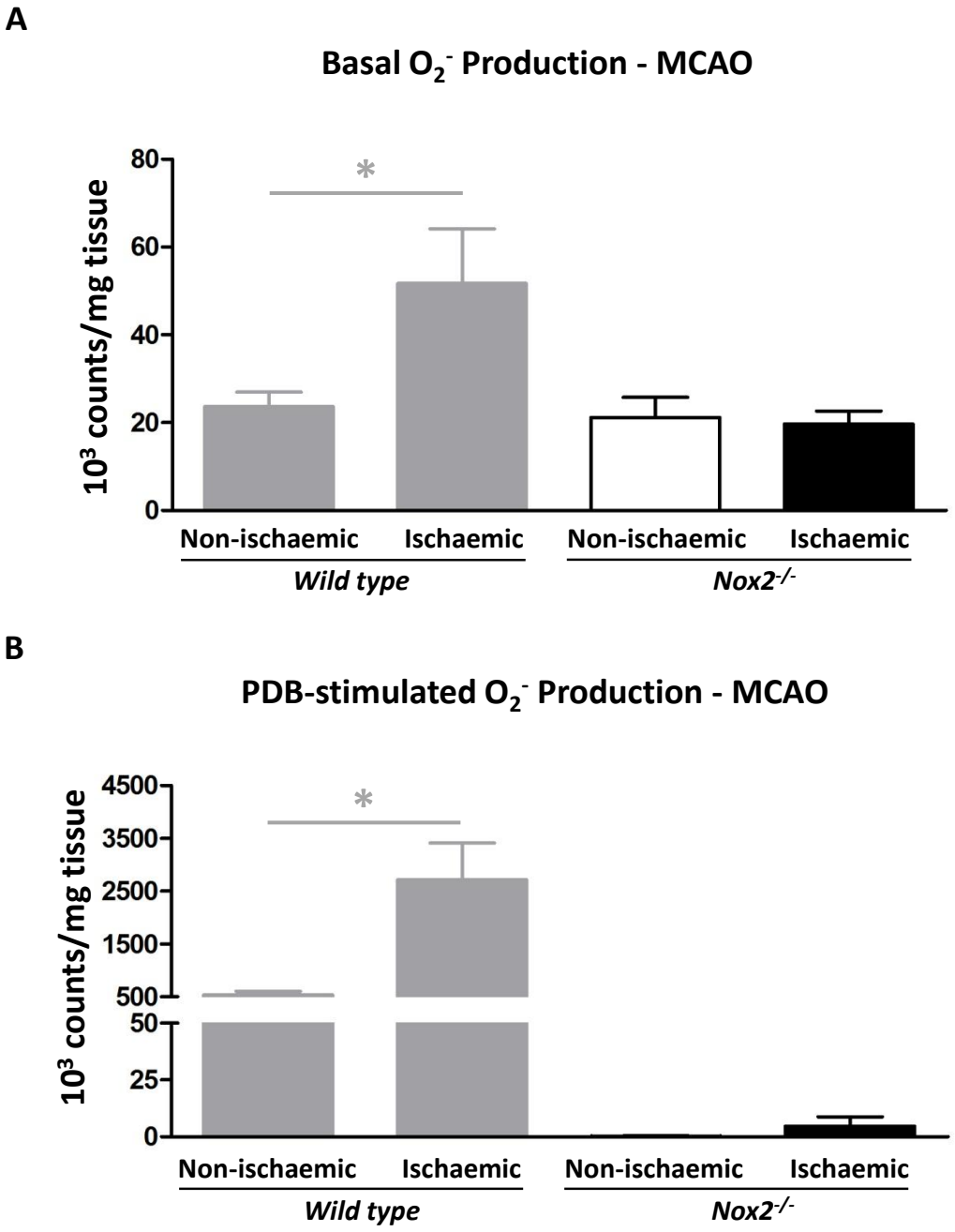


Figure 5. Basal **(A)** and phorbol-12, 13 dibutyrate (PDB; 10 µmol/L) -stimulated **(B)** O_2^- production by non-ischaemic and ischaemic middle cerebral arteries (MCA) from wild type and Nox2-deficient ($Nox2^{-/-}$) mice 24 h after cerebral ischaemia-reperfusion. O_2^- production was measured using 100 µmol/L L-012-enhanced chemiluminescence. The data for PDB-stimulated O_2^- production represents counts in PDB-treated arteries minus basal counts (i.e. in the absence of PDB). All results are expressed as 10^3 counts/mg of dry tissue weight and given as mean ± SEM (**A**, n=9 for both wild type groups and n=6 for both $Nox2^{-/-}$ groups; **B**, n=6 for both wild type groups and n=5 for non-ischaemic and n=6 for ischaemic $Nox2^{-/-}$ groups). ***P<0.05 vs. non-ischaemic MCA (paired t test). Data from wild type is reproduced from Figure 3A and 4A for comparative purposes.*

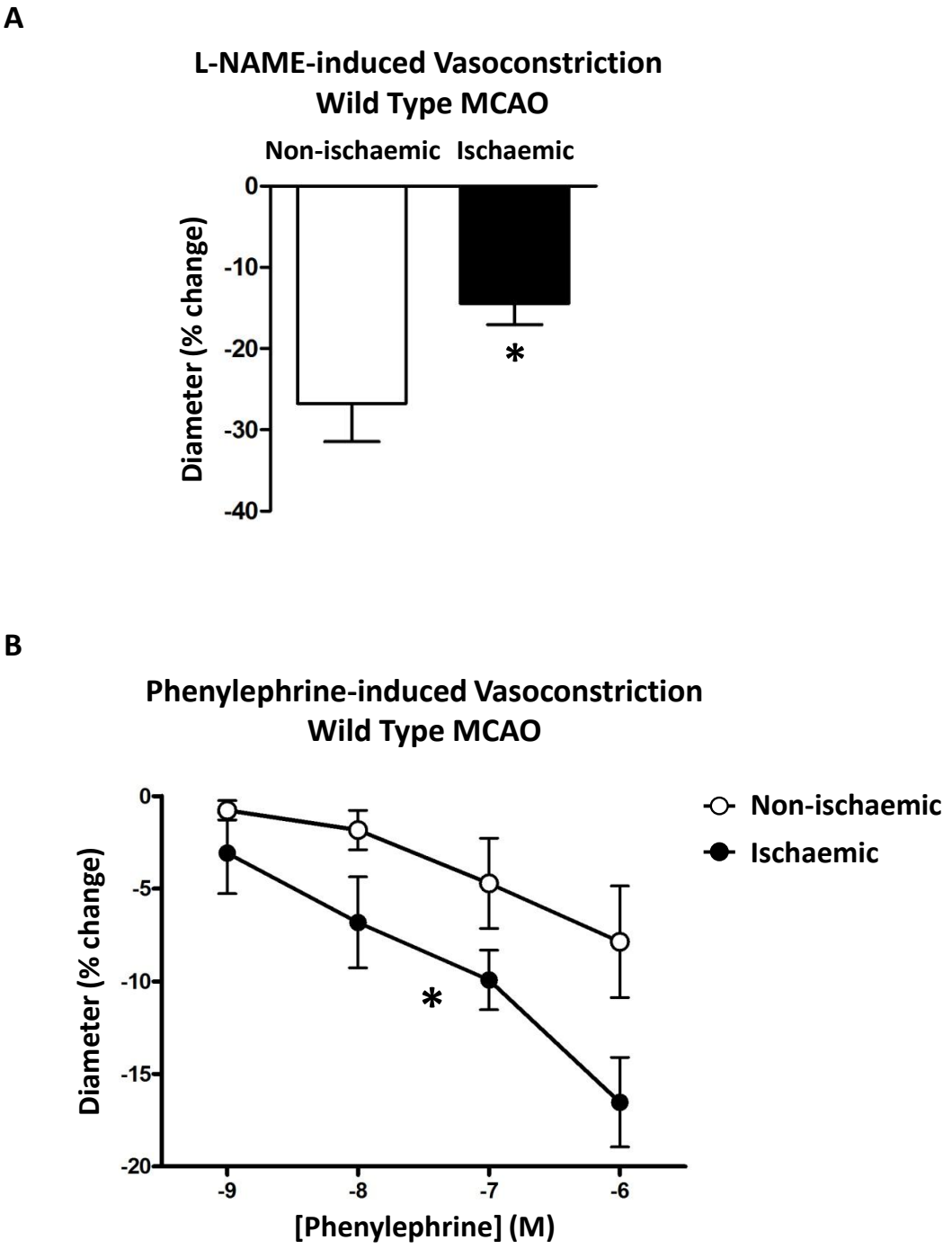


Figure 6. Constrictor responses of isolated non-ischaemic and ischaemic middle cerebral arteries (MCA) from wild type mice to the NOS inhibitor L-NAME (100 µmol/L; **A**) and α-adrenoceptor agonist phenylephrine (1 nmol/L – 1 µmol/L; **B**) 24 h after cerebral ischaemia-reperfusion. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (**A**, n=6 for non-ischaemic MCA and n=8 for ischaemic MCA; **B**, n=6 for non-ischaemic MCA and n=4 for ischaemic MCA). **(A)** **P*<0.05 vs. non-ischaemic MCA (unpaired *t* test), **(B)** **P*<0.05 vs. non-ischaemic MCA (two-way ANOVA with a Bonferroni multiple comparison post-hoc test).

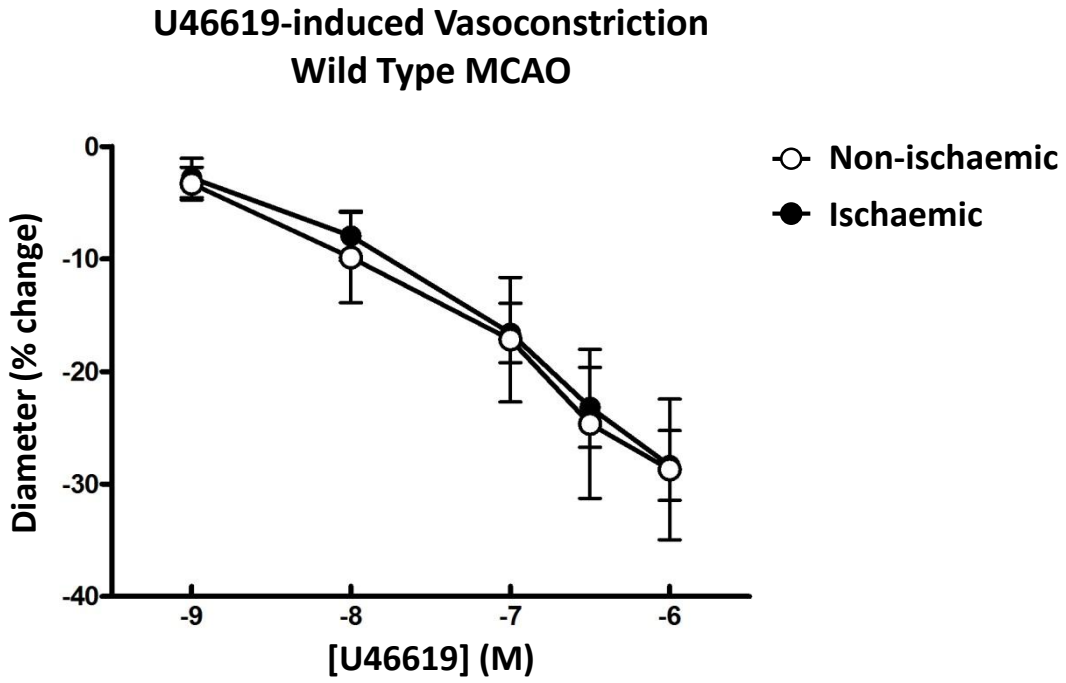


Figure 7. Constrictor responses of isolated non-ischaemic and ischaemic MCA from wild type mice to the thromboxane A_2 mimetic U46619 (1 nmol/L – 1 µmol/L) 24 h after cerebral ischaemia-reperfusion. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (n=7 for non-ischaemic MCA and n=6 for ischaemic MCA).

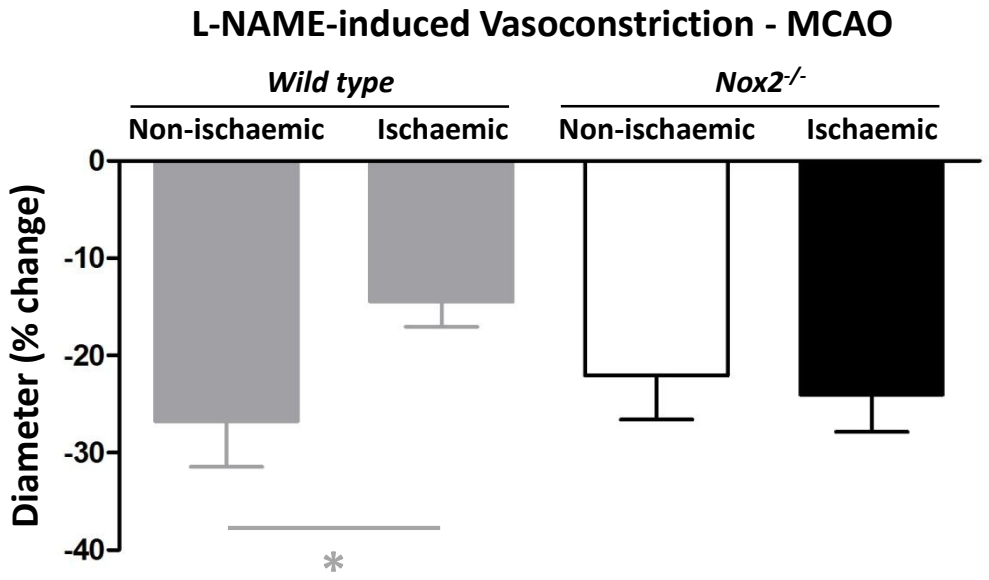


Figure 8. Constrictor responses of isolated non-ischaemic and ischaemic middle cerebral arteries (MCA) from wild type and Nox2-deficient ($Nox2^{-/-}$) mice to the NOS inhibitor L-NAME (100 µmol/L) 24 h after cerebral ischaemia-reperfusion. Results are expressed as % change in intraluminal diameter and given as mean ± SEM (wild type, n=6 for non-ischaemic MCA and n=8 for ischaemic MCA; $Nox2^{-/-}$, n=6 for both groups*). *P<0.05 vs. non-ischaemic MCA (unpaired t test). Data from wild type is reproduced from Figure 5 for comparative purposes.*

Discussion

The present study provides novel insight into the effects of cerebral ischaemia-reperfusion on the cerebral circulation. Specifically, the major findings of the present study are that following cerebral ischaemia-reperfusion, ischaemic MCA from wild type mice have: (1) elevated basal and PDB-stimulated O_2^- production and (2) reduced NO· function. Following cerebral ischaemia-reperfusion in Nox2$^{-/-}$ mice, elevated O_2^- production and impaired NO· function was abrogated. The findings of this study indicates that cerebral ischaemia-reperfusion results in augmented O_2^- production and impaired NO·-dependent signalling. These effects of cerebral ischaemia on cerebral artery function are dependent on Nox2-NADPH oxidase.

The brain has minimal energy stores and is therefore particularly sensitive to disruptions in blood flow. As such, cerebral blood vessels are highly specialised in order to deliver a constant supply of glucose and oxygen to the brain. Following cerebral ischaemia, the function and integrity of cerebral blood vessels are critical to support cerebral blood flow and thus minimise further neuronal injury and neurological impairment. The infarct core is the area subjected to the most severe ischaemia and subsequently undergoes necrotic cell death. Between the infarct core and unaffected neuronal tissue lies an area of constrained blood flow, referred to as the penumbra (Astrup *et al.*, 1981; Astrup *et al.*, 1977; Dirnagl *et al.*, 1999). Neurons within the penumbra die over a more prolonged period, which may extend from hours to days (Dirnagl *et al.*, 1999). Previous experimental studies have found that cerebrovascular ROS production is augmented during the first few hours of reperfusion (Kontos *et al.*, 1992; Mori *et al.*, 1999; Nelson *et al.*, 1992). This augmentation in ROS levels may result in further vascular dysfunction and a decline in perfusion of the ischaemic brain. More recently, it has been reported that NADPH (substrate for NADPH oxidase) -stimulated O_2^- production is elevated in cerebral arteries from the penumbra up to 72 h after mild stroke induced by transient MCA occlusion in rats (Miller *et al.*, 2006). These findings suggest that O_2^- production remains elevated long after the ischaemic insult and thus may participate in delayed neuronal damage within the penumbra. Furthermore, these findings suggest that NADPH oxidase may be a pathological source of O_2^- following cerebral ischaemia. The Nox2-containing isoform of NADPH oxidase has been shown to be an important source of augmented vascular O_2^-

production in a number of diseases affecting the cerebral circulation such as hypertension and hypercholesterolaemia (see Chapter 5, Girouard *et al.*, 2006). This raises the possibility that Nox2-NADPH oxidase may be the source of augmented vascular O_2^- production following cerebral ischaemia-reperfusion. However, no study has definitively tested the role of Nox2-NADPH oxidase in the augmented O_2^- production by cerebral arteries following cerebral ischaemia-reperfusion. To test this, we measured basal and PDB [Nox2-NADPH oxidase activator (Gupte *et al.*, 2009)] -stimulated O_2^- production by cerebral arteries from wild type and $Nox2^{-/-}$ mice following 24 h cerebral ischaemia-reperfusion. Using the chemiluminescence probe, L-012, we found that basal O_2^- production by MCA from the ischaemic cerebral hemisphere was approximately 2-fold greater than the MCA from the non-ischaemic cerebral hemisphere 24 h after cerebral ischaemia-reperfusion. Importantly, basal O_2^- production was similar between MCA from the left and right cerebral hemispheres (anatomically corresponding to non-ischaemic and ischaemic MCA) of sham-operated animals. Taken together, these findings suggest that cerebral ischaemia-reperfusion results in an augmentation of O_2^- production by MCA. Furthermore, PDB-stimulated O_2^- production by ischaemic MCA was approximately 6-fold greater than in non-ischaemic MCA. Consistent with basal O_2^- production, PDB-stimulated O_2^- production was similar between left and right MCA from sham operated mice. Thus, these findings suggest that Nox2-NADPH oxidase activity may be elevated following cerebral ischaemia-reperfusion.

It has previously been demonstrated that Nox2-NADPH oxidase deletion or inhibition improves outcome following cerebral ischaemia (Jackman *et al.*, 2009; Kahles *et al.*, 2007; Walder *et al.*, 1997; Wang *et al.*, 2006). Importantly, in the present study the rCBF profile during ischaemia and reperfusion was similar between wild type and $Nox2^{-/-}$ mice, suggesting that Nox2 deletion per se does not affect cerebral blood flow during ischaemia-reperfusion. Consistent with these previous reports, we found in that present study that compared with wild type mice, $Nox2^{-/-}$ mice had smaller cerebral infarct volumes, and improved neurological outcome following MCAO. There was also a trend for smaller cerebral oedema volume in $Nox2^{-/-}$ mice compared with wild type mice, however, this did not reach statistical significance. This last point notwithstanding and consistent with previous studies, these findings demonstrate that Nox2-NADPH oxidase plays a

detrimental role in cerebral ischaemia-reperfusion. While previous studies have identified Nox2-NADPH oxidase as a source of O_2^- in neuronal tissue, microglial cells and T-lymphocytes after cerebral ischaemia-reperfusion (Brait *et al.*, 2010; Chen *et al.*, 2009; Kunz *et al.*, 2006; McCann *et al.*, 2008), it is currently unclear if Nox2-NADPH oxidase contributes to augmented O_2^- production in the cerebral vasculature after ischaemia-reperfusion. Therefore, we next studied cerebral vessels from $Nox2^{-/-}$ mice after MCAO surgery. In $Nox2^{-/-}$ mice, basal O_2^- production was similar in non-ischaemic and ischaemic MCA. Furthermore, PDB-stimulated O_2^- production was virtually absent in both non-ischaemic and ischaemic MCA from $Nox2^{-/-}$ mice. These findings indicate that O_2^- production was not elevated in ischaemic MCA from $Nox2^{-/-}$ mice following MCAO. Overall, we demonstrate, for the first time, that Nox2-NADPH oxidase is the key source of the augmented O_2^- production by ischaemic MCA following cerebral ischaemia-reperfusion.

Previous studies have reported impaired NO·-dependent vasorelaxation during a number of disease states affecting the cerebral circulation, including hypercholesterolaemia and hypertension (see Chapter 5, Capone *et al.*, 2010; Chrissobolis *et al.*, 2010; Kazama *et al.*, 2003; Kitayama *et al.*, 2007). Furthermore, it has been reported that ischaemia-reperfusion has profound effects on NO· function in cerebral blood vessels. Indeed, it has been reported that NO·-dependent dilatation of pial arterioles from mice are impaired during bilateral carotid artery ligation induced ischaemia (Rosenblum *et al.*, 1995). Furthermore, NO·-dependent relaxation of feline and rat cerebral arteries is impaired up to 24 h after cerebral ischaemia-reperfusion (Cipolla *et al.*, 1997; Clavier *et al.*, 1994; Mayhan *et al.*, 1988; Nelson *et al.*, 1992; Rosenblum *et al.*, 1997). Moreover, it has also been reported that basal NO· production, as measured by constriction to L-NAME, in cerebral arteries from rats is impaired 24 h after ischaemia-reperfusion (Cipolla *et al.*, 2008; Cipolla *et al.*, 2009; Marrelli *et al.*, 1999). Taken together, these findings indicate that cerebral ischaemia-reperfusion is associated with impaired NO·-dependent vasorelaxation. However, it is currently unknown if augmented O_2^- production by Nox2-NADPH oxidase causes impaired NO· function in mice following cerebral ischaemia-reperfusion. To test this, we measured constrictor responses of non-ischaemic and ischaemic MCA from wild type and $Nox2^{-/-}$ mice to the NOS inhibitor

L-NAME. Consistent with the aforementioned studies, constriction of isolated ischaemic MCA from wild type mice to L-NAME were substantially smaller than non-ischaemic MCA. Within the vasculature, it has been demonstrated that NO· normally offsets vasocontraction to α-adrenoceptor agonists, such as phenylephrine, as NOS inhibition augments vasocontraction to phenylephrine(Dora *et al.*, 2000; Lopez *et al.*, 2010). A recent study of MCA from goats following cerebral ischaemia-reperfusion demonstrated that contraction to α-adrenoceptor agonists, including phenylephrine, are augmented (Monge *et al.*, 2010). Consistent with this finding, we found in the present study that constrictor responses to phenylephrine were greater in ischaemic MCA from wild type mice compared with non-ischaemic MCA. These findings are consistent with the concept that NO· function is impaired after cerebral ischaemia-reperfusion. In contrast to phenylephrine and L-NAME, constrictor responses to the thromboxane A_2 (TXA_2) mimetic, U46619, were similar between non-ischaemic and ischaemic MCA. Within the vasculature, U46619 activates the TXA_2 receptor, ultimately leading to the activation of a number protein kinases such as protein kinase C and Rho-kinase, which results in phosphorylation of myosin light chain and smooth muscle contraction (Nakahata, 2008). As U46619-induced constriction of ischaemic and non-ischaemic MCA were similar, it suggests that the contractility of the MCA is not altered following ischaemia-reperfusion, but rather NO· function is impaired post-ischaemia. In order to determine if Nox2-NADPH oxidase-derived ROS contributed to impaired NO· function in wild type mice following MCAO, we next measured L-NAME-induced constrictor responses in $Nox2^{-/-}$ mice following MCAO. L-NAME-induced constrictor responses were similar between non-ischaemic and ischaemic MCA from $Nox2^{-/-}$ mice, suggesting that NO· function is not impaired in $Nox2^{-/-}$ mice. While we have not tested whether impaired NO· function in wild type mice could be restored by O_2^- scavengers such as superoxide dismutase (SOD) or tempol, it is conceivable that Nox2-NADPH oxidase-derived O_2^- may acutely scavenge NO· in ischaemic MCA following cerebral ischaemia-reperfusion. Indeed, Nelson *et* al., (1992) demonstrated that NO·-dependent vasorelaxation responses could be improved with SOD + catalase treatment (Nelson *et al.*, 1992), suggesting that O_2^- may be acutely scavenging NO· following cerebral ischaemia-reperfusion (Nelson *et al.*, 1992). In contrast, it has also been reported that impaired acetylcholine-induced vasorelaxation following ischaemia-reperfusion is not improved by SOD + catalase treatment (Rosenblum *et al.*, 1997).

Furthermore, in a rat model of haemorrhagic stroke, pre- or post-stroke treatment with the SOD mimetic, tempol, failed to improve impaired NO·-function in MCA (Daneshtalab *et al.*, 2010). Currently, the reason(s) for these conflicting findings are unclear, and further studies are needed to determine if ROS scavengers are beneficial in improving NO· function following cerebral ischaemia. However, the findings of the present study provide strong evidence to suggest that excessive production of O_2^- by Nox2-NADPH oxidase plays a key role in impaired NO· function 24 h after cerebral ischaemia. Furthermore, these findings may suggest that inhibiting the source of augmented O_2^- production following cerebral ischaemia may be a more effective treatment strategy than treatment with ROS scavengers.

As discussed above, NO· is an important endogenous vasodilator in cerebral arteries. In addition to its vasodilator effects, NO· is a well known inhibitor of platelet aggregation (Bermejo *et al.*, 2005; Mondoro *et al.*, 2001) and inhibits leucocyte adhesion and recruitment into the vessel wall (Ahluwalia *et al.*, 2004; Kubes *et al.*, 1991). It is well established that stroke induces a systemic inflammatory response (Hurn *et al.*, 2007; Offner *et al.*, 2006) and leucocyte rolling and adhesion is elevated in the vasculature following cerebral ischaemia (Ishikawa *et al.*, 2004; Yilmaz *et al.*, 2006). Furthermore, it has been reported that immune cells, particularly T-lymphocytes, enter the brain after ischaemia-reperfusion and contribute to brain injury (Brait *et al.*, 2010; Hurn *et al.*, 2007; Yilmaz *et al.*, 2006). As such, reducing immune cell infiltration into the brain could potentially reduce ischaemia-reperfusion induced brain damage. NO· has been shown to reduce leucocyte recruitment via the down-regulation of adhesion molecules, such as P-selectin (Ahluwalia *et al.*, 2004). Therefore, improving NO· bioavailability after cerebral ischaemia would conceivably be of benefit by increasing cerebral blood flow to ischaemic/penumbral brain areas, reducing immune cell adhesion/infiltration as well as reducing the likelihood of secondary thrombus formation and secondary stroke. However, post stroke therapies that increase NO· levels, without reducing O_2^- levels, may not be an effective treatment strategy as the NO· may be scavenged by O_2^-, generating $ONOO^-$, which has been shown be a mediator of blood brain barrier dysfunction following cerebral ischaemia-reperfusion (Gursoy-Ozdemir *et al.*, 2000; Gursoy-Ozdemir *et al.*, 2004). As such, inhibiting vascular Nox2-NADPH oxidase, thus reducing augmented

vascular O_2^- production, after cerebral ischaemia would conceivably improve NO· bioavailability, increase cerebral blood flow, reduce the risk of secondary stroke and potentially improve functional outcomes.

CHAPTER 7

GENERAL DISCUSSION

Summary of the Major Findings

Collectively, the findings of this thesis provide novel insight into the role of Nox2-NADPH oxidase-derived reactive oxygen species in the regulation of cerebral vascular function during health and disease. Specifically, the major findings of this thesis are as follows:

Chapter 3: Gender Influences Cerebrovascular Responses to Angiotensin II Through Nox2-Derived Reactive Oxygen Species

One major new finding of this study is that gender influences cerebral vascular responses to angiotensin II by modulating reactive oxygen species (ROS) production by Nox2-NADPH oxidase. Specifically, we found that angiotensin II-stimulated superoxide (O_2^-) and hydrogen peroxide (H_2O_2) production by cerebral arteries from male wild type mice was ≥ 2-fold greater compared with cerebral arteries from female mice. Furthermore, the relatively lower levels of ROS production in females were associated with smaller constrictions of middle cerebral arteries (MCA) to angiotensin II *in vitro*. In cerebral arteries from male Nox2-deficient ($Nox2^{-/-}$) mice, angiotensin II-stimulated O_2^- production and vasoconstriction were significantly attenuated such that responses were comparable to those found in cerebral arteries from female wild type mice. By contrast, Nox2 deficiency had no effect on ROS production and constrictor responses in female mice. Importantly, our findings indicate that such gender-related effects on angiotensin II-stimulated responses are unlikely to be attributable to 1) greater expression of Nox2 or differential cellular localization; 2) differing rates of O_2^- inactivation by superoxide dismutases (SOD) and/or nitric oxide; or 3) differential signalling of angiotensin II through angiotensin type 1 and 2 receptors (AT_1 and AT_2 receptors). Thus, future studies are needed to fully elucidate the molecular basis for the higher angiotensin II-stimulated Nox2 activity in the cerebral circulation of males. This latter point notwithstanding, the findings of this study provide the first direct evidence that Nox2-NADPH oxidase plays a key role in generating ROS and mediating constrictor responses in cerebral arteries of males but not females. Moreover, these findings provide a mechanistic basis for the gender-dependent difference in cerebral vascular responses to angiotensin II.

Chapter 4: Role of Hydrogen Peroxide in Angiotensin II-Induced Constriction of Cerebral Arteries

Having demonstrated a key for Nox2-derived ROS in mediating constrictor responses to angiotensin II in the cerebral circulation male mice, we next used a pharmacological approach to determine the role(s) of O_2^- and/or H_2O_2 in mediating this effect. Using the ROS scavengers, tempol (SOD mimetic) and EUK-134 (SOD/catalase mimetic) we demonstrate for the first time that H_2O_2 mediates vasoconstriction to angiotensin II in the mouse cerebral circulation. Specifically, we found that tempol potentiated constrictions of MCA to angiotensin II and significantly increased angiotensin II-stimulated H_2O_2 levels in cerebral arteries. By contrast, EUK-134 virtually abolished contractile responses to angiotensin II and significantly decreased angiotensin II-stimulated H_2O_2 levels. It has previously been reported that angiotensin II constricts basilar arteries via activation of the RhoA/Rho-kinase pathway. Furthermore, our findings suggest that angiotensin II-stimulated H_2O_2 activates the RhoA/Rho-kinase pathway to elicit constriction of mouse MCA. Taken together with our findings in Chapter 3, the findings of this chapter indicate that angiotensin II-induced constriction of MCA is predominantly mediated by Nox2-NADPH oxidase-derived H_2O_2, possibly via the activation of RhoA/Rho-kinase pathway.

Chapter 5: Augmented Superoxide Production by Nox2-NADPH Oxidase Causes Cerebral Artery Dysfunction During Hypercholesterolaemia

Interestingly, we found no evidence for atherosclerotic lesions or fatty streaks in cerebral arteries from hypercholesterolaemic apoliprotein E-deficient ($ApoE^{-/-}$) or $Nox2^{-/-}/ApoE^{-/-}$ animals. Despite the lack of lesions, O_2^- levels generated by cerebral arteries from $ApoE^{-/-}$ mice were approximately 2-fold higher than in wild type, and importantly, $Nox2^{-/-}/ApoE^{-/-}$ mice, suggesting Nox2-NADPH oxidase is the source of augmented O_2^- levels. The augmented O_2^- levels in hypercholesterolaemic $ApoE^{-/-}$ mice were associated with impaired constrictor responses to the NOS inhibitor L-NAME, suggesting basal NO· function was impaired. Consistent with the O_2^- data, L-NAME-induced constrictions were comparable with wild type mice, indicating NO· function is normal in the absence of Nox2-NADPH oxidase. Using the SOD mimetic tempol, we provide evidence to suggest that O_2^- acutely scavenges NO·, reducing its bioavailability. The augmented O_2^- levels were not associated changes in the expression level of the Nox2 catalytic subunit. However,

protein expression of the Nox2-NADPH oxidase organiser subunit, p47phox was approximately 2-fold higher in cerebral arteries from ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ compared with wild type mice. Furthermore, cerebrovascular protein expression of SOD isoforms 1-3 and eNOS were similar between all genotypes. Interestingly, at this time point hypercholesterolaemia may induce an inflammatory phenotype within the cerebral vasculature. Specifically, protein expression of monocyte chemoattractant protein 1 (MCP-1) was similarly elevated in cerebral arteries from ApoE$^{-/-}$ and Nox2$^{-/-}$/ApoE$^{-/-}$ compared with wild type. Overall, the main findings of this chapter indicate that despite the lack of atherosclerotic lesions, the activity of Nox2-NADPH oxidase is greater in cerebral arteries from hypercholesterolaemic animals, which is likely due to increased expression of p47phox. Furthermore, we provide evidence to suggest that the elevated Nox2-NADPH oxidase-derived O_2^- acutely scavenges NO·, reducing its bioavailability.

Chapter 6: Effect of Transient Cerebral Ischaemia on Middle Cerebral Artery Function: Role of Nox2-NADPH Oxidase

Twenty-four hours after cerebral ischaemia-reperfusion, basal O_2^- production by ischaemic MCA was approximately 2-fold higher compared with non-ischaemic MCA from wild type mice. Furthermore, phorbol-12, 13-dibutyrate (PDB; Nox2-NADPH oxidase activator) –stimulated O_2^- production was approximately 6-fold higher in ischaemic versus non-ischaemic MCA from wild type mice. Following cerebral ischaemia-reperfusion, constrictor responses to the NOS inhibitor L-NAME were significantly attenuated in ischaemic MCA compared with non-ischaemic MCA, suggesting NO· function is selectively impaired in ischaemic MCA. By contrast, constrictor responses to the thromboxane A_2 mimetic U46619 were similar between ischaemic and non-ischaemic MCA. Thus, 24 h after cerebral ischaemia-reperfusion, ischaemic MCA generate significantly more O_2^- and this is associated with impaired NO· function.

As has been previously reported, neurological outcome and total infarct volume was reduced in Nox2$^{-/-}$ mice compared with wild type mice. We report, for the first time, that Nox2-NADPH oxidase is the sole source of augmented O_2^- production 24 h after cerebral ischaemia-reperfusion. Specifically, both basal and PDB-stimulated O_2^- production was not elevated in ischaemic MCA from Nox2$^{-/-}$ mice. Moreover, basal NO· function was

similar between ischaemic and non-ischaemic MCA from Nox2$^{-/-}$ mice following MCAO, suggesting normal NO· function in the absence of Nox2-NADPH oxidase. Thus, the main findings of this chapter suggest that cerebral ischaemia-reperfusion results in augmented levels of Nox2-NADPH oxidase-derived O_2^- which impairs NO· function. Considering our previous findings in hypercholesterolaemic mice (Chapter 5), NO· function may be impaired as a result of acute scavenging of NO· by O_2^-.

Potentially Consequences of Chronically Augmented Nox2-derived ROS Levels During Cerebrovascular Disease

One of the major findings of the studies in this thesis is that Nox2-NADPH oxidase-dependent ROS production by cerebral arteries can be acutely increased by humoral stimuli (i.e. angiotensin II), as well as being chronically augmented during disease (hypercholesterolaemia and after cerebral ischaemia-reperfusion). Moreover, our findings would suggest that when the activity Nox2-NADPH oxidase is augmented within cerebral arteries this is likely to lead to excessive ROS production and impaired NO· function. Such impairment in NO· function may occur as a result of the acute reaction of NO· with O_2^-. Indeed, in the setting of hypercholesterolaemia we found that impaired NO· function in cerebral arteries could be acutely reversed by the O_2^- scavenger tempol. Importantly, the reaction of O_2^- with NO· not only decreases NO· bioavailability but also results in the generation of the highly reactive molecule peroxynitrite ($ONOO^-$). $ONOO^-$, generated endogenously by Nox2 NADPH oxidase-derived O_2^- and eNOS-derived NO·, has been shown to impair functional hyperaemia and endothelium-dependent dilatation of cerebral arterioles (Girouard *et al.*, 2007). Furthermore, $ONOO^-$ has been reported to impair cerebrovascular autoregulation (DeWitt *et al.*, 2001) and may also contribute to microvascular dysfunction resulting in oedema formation following cerebral ischaemia-reperfusion (Gursoy-Ozdemir *et al.*, 2004). Thus, evidence from studies in cerebral arteries indicates that $ONOO^-$ is a critical mediator of cerebral artery damage during oxidative stress. In non-cerebral arteries, $ONOO^-$ has been reported to decrease NO· production via oxidation of the NOS cofactor tetrahydrobiopterin (BH_4) and the zinc thiolate complex in eNOS, leading to uncoupling of eNOS and O_2^- generation (Chen *et al.*, 2010; Landmesser *et al.*, 2003; Zou *et al.*, 2002). While it remains to be tested, the effects

of excessive Nox2-NADPH oxidase-derived ROS on cerebrovascular function could extend beyond acute scavenging of NO·.

In addition to effects on NO· bioavailability, augmented Nox2-NADPH oxidase-derived ROS may have direct effects on the cerebral circulation. Indeed, findings in Chapters 3 and 4 indicate that Nox2-NADPH oxidase-derived H_2O_2 mediates constrictor responses to angiotensin II via the RhoA/Rho-kinase pathway. Therefore, during diseases associated with augmented Nox2-NADPH oxidase-derived ROS production, cerebrovascular tone may be elevated indirectly via reducing NO· bioavailability and directly via the activation of vasoconstrictor pathways, such as the RhoA/Rho-kinase pathway. Furthermore, it has been reported that high levels of exogenous ROS cause apoptosis of cerebrovascular cells (Li *et al.*, 2003a; Li *et al.*, 2003b), which may also contribute to impaired cerebrovascular function during disease. Additionally, in the systemic circulation, it has been demonstrated that increased levels of vascular Nox2-NAPDH oxidase-derived ROS during atherosclerosis upregulates the expression of adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Vendrov *et al.*, 2007). These adhesion molecules are believed to play a critical role in the recruitment of leucocytes into the systemic vasculature (Vendrov *et al.*, 2007). Therefore, if similar processes apply within the cerebral circulation, it is possible that during diseases where Nox2-NADPH oxidase-derived ROS levels are augmented, there is an increase in the expression of adhesion molecules, and thus, recruitment of immune cells into the vasculature. As discussed previously, immune cells have been implicated in mediating vascular dysfunction in the systemic circulation during hypertension and hypercholesterolaemia (Guzik *et al.*, 2007; Stokes *et al.*, 2007a) and neurological damage following stroke (Hurn *et al.*, 2007; Yilmaz *et al.*, 2006). However, it is currently unknown if similar mechanisms contribute to cerebrovascular dysfunction during such diseases. Therefore, chronically augmented Nox2-NADPH oxidase-derived ROS may have both indirect and direct, deleterious effects on the cerebral circulation via increasing cerebrovascular tone as well as inducing apoptosis and inflammation during diseases where a role for cerebrovascular Nox2-NADPH oxidase has been shown. This may result in an increase in cerebrovascular tone and reduced overall perfusion of the brain. Furthermore, functional hyperaemia may be impaired due to increased O_2^- and reduced

NO·, which may lead to insufficient blood flow to active neurons and potentially to cognitive impairment.

Nox2-NADPH Oxidase and Gender-dependent Differences in Diseases Affecting the Cerebral Circulation

Hypertension is a major risk factor for ischaemic (both thrombotic and lacunar) and haemorrhagic stroke, as well as impaired cognitive function (Jackson *et al.*, 2010; O'Donnell *et al.*, 2010; Wolf *et al.*, 2007). Interestingly, the clinical incidence of cerebrovascular diseases such as stroke, is lower in premenopausal females compared with males and postmenopausal females (Appelros *et al.*, 2009; Prencipe *et al.*, 1997). As discussed in Chapter 1 and 3, it is likely that numerous mechanisms contribute to the gender-dependent differences in the clinical incidence of such diseases. However, the findings of this thesis raise the intriguing possibility that gender-dependent differences in Nox2-NADPH oxidase-dependent ROS production may play an important role. As discussed, we have demonstrated that Nox2-NADPH oxidase plays a major role in mediating cerebral artery responses to angiotensin II in non-diseased male but not female mice, suggesting that Nox2-NADPH oxidase activity is greater in the cerebral circulation of males under physiological conditions. Thus, it is conceivable that because of their higher physiological levels of Nox2-NADPH oxidase-derived ROS, the increase in ROS to reach pathological levels of ROS during disease states (e.g. hypertension, hypercholesterolaemia and following ischaemia and reperfusion) may be less in males compared with females, making males more susceptible to the development of cerebrovascular dysfunction and possibly cerebrovascular disease. Consistent with this hypothesis is the recent finding that chronic angiotensin II-induced hypertension impairs NO·-dependent vasodilatation in males but not females (Girouard *et al.*, 2008). The cerebrovascular protection afforded by the female gender has been shown to be due to the effects of oestrogen (Capone *et al.*, 2009; Girouard *et al.*, 2008; Miller *et al.*, 2007b). Therefore, it is conceivable that in diseases where Nox2-NADPH oxidase-derived ROS is detrimental, such as during hypercholesterolaemia, hypertension and following cerebral ischaemia-reperfusion, cerebral arteries from premenopausal females may be relatively spared from cerebrovascular dysfunction. This may underlie the lower clinical incidence

of cerebrovascular disease observed in premenopausal females compared with males and postmenopausal females. Clearly, future studies are needed to fully determine whether lower Nox2-NADPH oxidase activity in premenopausal females in fact protects against the initiation and development of cerebral vascular dysfunction associated with cerebral vascular disease.

Nox2-NADPH Oxidase and the Effects of Hypercholesterolaemia on the Cerebral Circulation

In contrast to other 'high risk' cardiovascular states such as hypertension, our overall understanding of the effects of hypercholesterolaemia on cerebrovascular function is limited. Moreover, as discussed previously, the relationship between cholesterol levels and stroke incidence is unclear. The findings of this thesis provide novel insight into the mechanistic effects of hypercholesterolaemia on cerebrovascular reactivity. Indeed, our findings indicate that despite the absence of atherosclerotic lesions, cerebral arteries from hypercholesterolaemic mice have elevated ROS production and impaired NO· function, as a result of higher Nox2-NADPH oxidase activity. Thus, Nox2-NADPH oxidase plays a central role in mediating cerebral vascular dysfunction during hypercholesterolaemia. Future studies are needed to determine whether such changes increase the risk of ischaemic stroke or if they worsen outcome following a stroke. As discussed earlier, it is plausible that the loss of NO· during hypercholesterolaemia would lead to dysregulation of cerebral blood flow as a result of impaired functional hyperaemia and/or via vascular remodelling. This may result in the reduced cerebral perfusion and as such, may result in the brain being at risk of an ischaemic event. Furthermore, following an ischaemic event, stroke outcome may be worsened due to compromised cerebral blood flow owing to reduced NO· function following stroke. While we demonstrate a role for Nox2-NADPH oxidase in the cerebrovascular dysfunction during hypercholesterolaemia, the mechanisms by which elevated plasma cholesterol levels induce cerebrovascular dysfunction are currently unknown. As discussed above, it is possible that infiltrating immune cells are the mediators of cerebrovascular dysfunction during hypercholesterolaemia. However, further studies are needed to determine if this or other factors contribute to the initiation of hypercholesterolaemia-induced cerebral

artery dysfunction. Furthermore, as cerebral arteries did not develop atherosclerotic lesions during hypercholesterolaemia, investigation of the mechanisms that contributes to this may lead to the development of therapies for preventing or reducing atherosclerosis in the systemic circulation.

Nox2-NADPH Oxidase and Cerebrovascular Dysfunction Caused by Ischaemia-Reperfusion

Currently, there is only one drug approved for treatment of ischaemic stroke, tissue plasminogen activator (tPA). However, tPA can only be administered within 4.5 h after the onset of ischaemia and only a small proportion of ischaemic stroke patients are treated. Indeed, a recent study of over 5000 ischaemic stroke patients reported that only 12.7 % were treated with tPA (van Wijngaarden *et al.*, 2009). As such, there is much need to develop novel strategies for acute and chronic stroke therapy. As discussed previously in this thesis, cerebral ischaemia-reperfusion has been reported to increase vascular ROS production and impair NO· function within minutes of the onset of reperfusion (Mayhan *et al.*, 1988; Mori *et al.*, 1999; Nelson *et al.*, 1992). Furthermore, it has been shown that the elevated ROS production persists for several days after the ischaemic insult (Miller *et al.*, 2006b). The key finding of this study is that at 24 h after the ischaemic insult, the augmented ROS production and impaired NO· function is completely dependent on Nox2-NADPH oxidase and likely occurs as a result of increased Nox2-NADPH oxidase activity. As discussed in Chapter 6, the penumbral brain tissue is potentially salvageable. Thus, we predict that improving NO· bioavailability after cerebral ischaemia may result in improved cerebral blood flow and possibly a reduction in infarct size due to improved perfusion of the penumbra. Furthermore, increasing NO· bioavailability is likely to decrease the chance on secondary thrombus formation due to reduced platelet aggregability. Moreover, immune cell infiltration is likely to be reduced due to a suppression of adhesion molecule expression. All of these beneficial aspects suggest that increasing cerebrovascular NO· levels is a potential stroke therapy. However, as discussed in Chapter 6, excessive levels of O_2^-/$ONOO^-$ may be a cause of damage during reperfusion and improving NO· levels should be accompanied with reducing O_2^- levels (see below). Clearly, future studies are needed to identify vascular protective agents and determine if they improve neurological

outcome alone, or when given in concert with neuroprotective agents. Furthermore, it is possible that part of the reason for the failure of neuroprotective agents in clinical trials has been that the cerebral vasculature is damaged following ischaemic stroke and is unable to meet the demands of neurons recovering from ischaemia.

Therapeutic Strategies for Limiting Augmented ROS Cerebral Vascular Levels

Overall, the findings of this thesis indicate that excessive ROS production by Nox2-NADPH oxidase may lead to diminished NO· function during disease. Specifically, Nox2-NADPH oxidase is the source of augmented ROS in cerebral arteries from: (1) males in response to the hypertensive mediator angiotensin II; (2) during hypercholesterolaemia; and (3) following cerebral ischaemia-reperfusion. Furthermore, the result of this thesis suggest that excessive production of ROS by cerebrovascular Nox2-NADPH oxidase is likely to be a cause of impaired NO· function. Over the last decade, there has been enormous interest in developing strategies to limit pathogenic ROS. However, it is important to remember that relatively low levels of ROS are likely to serve as cell signalling molecules for the regulation of normal cerebral vascular function. Furthermore, Nox2-NADPH oxidase is a critical component of host defence. As such, it is important that therapeutic strategies that alleviate the burden of oxidative stress only target pathological levels of ROS generated within the vasculature and thus preserve normal ROS-mediated cell signalling.

Antioxidants

In addition to the findings detailed in this thesis, a large body of clinical and experimental evidence support the concept that antioxidant therapy would be beneficial in limiting the damaging effects of ROS within the cerebral circulation. However, the results of the majority of large-scale clinical trials of antioxidant supplementation in vascular disease have yielded negative results. For example, the Heart Protection Study examined the effect of antioxidant supplementation (vitamin C, vitamin E and β-carotene) on vascular events in over 20,000 high risk patients (MRC/BHF, 2002). Although the plasma concentration of antioxidants increased, there was no difference in the incidence of vascular events, such as stroke (either ischaemic or haemorrhagic) (MRC/BHF, 2002). The

reasons for these disappointing findings may relate to the prescription of suboptimal doses of vitamins. Indeed, vitamins are weak antioxidants and it is known that high physiological concentrations of vitamins are required to effectively outcompete NO· for O_2^- (Jackson *et al.*, 1998; Sherman *et al.*, 2000). Alternatively, it is possible that intervention occurs to late in the progression of disease or that they exert pro-oxidant effects once converted to radical species after scavenging O_2^-. Thus, despite experimental promise, antioxidant therapy may be of little clinical benefit in improving endothelial function in diseases associated with oxidative stress.

Antioxidant Gene Therapy

A second possible method for limiting cerebrovascular ROS levels during disease is to increase the antioxidant capacity of the cerebral arteries via gene therapy. Studies of systemic arteries have shown that antioxidant gene therapy (e.g. SOD2, SOD3, catalase and glutathione peroxidise [GPx]) improves endothelial function in animal models of hypertension and hypercholesterolaemia (Chu *et al.*, 2003; Fennell *et al.*, 2002; Zanetti *et al.*, 2001). Furthermore, it has been shown that gene transfer of GPx protects the brain from ischaemic stroke damage (Hoehn *et al.*, 2003). While experimental evidence suggests that antioxidant gene therapy may be of potential clinical benefit, it has been suggested that its use may be hampered by several factors, including the limited efficacy of current delivery methods and the potential for vectors to evoke an immune response (Levonen *et al.*, 2008).

Inhibition of Nox2-NADPH Oxidase

The findings presented throughout this thesis, together with the wider literature, suggest that Nox2-NADPH oxidase is a deleterious source of ROS within the vasculature. Considering the failure of antioxidants and the limiting factors of antioxidant gene therapy, direct inhibition of Nox2-NADPH oxidase may be the best option for reducing Nox2-NADPH oxidase-derived ROS. Over the past decade, numerous inhibitors of NADPH oxidase have been developed, including diphenyleneiodonium (DPI), apocynin and gp91ds-tat. However, all of these drugs have a number of non-specific effects that would prevent their clinical usefulness. For example, DPI is a flavin antagonist and thus would inhibit all isoforms of NADPH oxidase in addition to other flavin containing enzymes, such

as NO· synthases. Apocynin and gp91ds-tat are Nox2-NADPH oxidase assembly inhibitors and are believed to prevent the binding of p47phox with Nox2 (Meyer *et al.*, 1999; Rey *et al.*, 2001). Experimentally, apocynin has been shown to be protective in ischaemic stroke (Jackman *et al.*, 2009a; Tang *et al.*, 2007; Wang *et al.*, 2006) and gp91ds-tat has been extensively shown to inhibit vascular O_2^- production (Capone *et al.*, 2010b; Miller *et al.*, 2005; Rey *et al.*, 2001)**.** However, while currently available NADPH oxidase inhibitors are valuable experimental tools and have greatly advanced our knowledge of this enzyme system, in their current form they are of limited clinical potential as they will target both vascular and immune cell Nox2-NADPH oxidase. As such, treatment with currently available Nox2-NADPH oxidase inhibitors is likely to render the patient immunocompromised. Therefore, more work is needed to identify possible structural or regulatory differences between vascular and immune cell Nox2-NADPH oxidase. The ultimate goal would be to develop an inhibitor that would inhibit the pathogenic production of ROS by vascular Nox2-NADPH oxidase without affecting immune cell Nox2. However, if immune cells are mediators of cerebrovascular dysfunction, inhibitors of immune cell Nox2 may be beneficial. Clearly, more work is needed in order to determine if vascular or immune cell Nox2-NADPH oxidase is the source of pathological levels of ROS in cerebral arteries before Nox2-NADPH oxidase can targeted therapeutically.

Limitations

With the exception of Chapter 5, all experiments were performed in young, healthy animals. In humans, the incidence of many diseases increases with age and patients often have co-morbidities. However, the use of non-diseased animals in Chapter 3, 4 and 6 allows us to investigate the specific effects of each disease state in isolation. It would be of interest, and of clinical relevance, to study cerebrovascular function in animals with multiple morbidities and/or at older age.

A second possible limitation is that all experiments were carried out *ex vivo*, and in relatively large cerebral arteries. *In vivo*, neurons and glial cells may have an important influence on cerebral artery tone, a phenomenon that we cannot replicate *ex vivo*. Also, it would be of interest to determine if Nox2-NADPH oxidase similarly influences cerebral

artery function in smaller arterioles and capillaries, which are the important interface between the circulation and brain parenchyma. Nevertheless, it is vital that we understand the effects of hypertension, hypercholesterolaemia and cerebral ischaemia to name but a few diseases, on both large and small cerebral artery function.

A third possible limitation particularly applies to the experiments in Chapter 6. We have only examined vascular O_2^- production and NO· function at 24 h after cerebral ischaemia. While it has been shown that cerebral artery O_2^- production is still elevated at 72 h after mild cerebral ischaemia in rats (Miller *et al.*, 2006b), it is currently unclear if Nox2-NADPH oxidase is the source of O_2^- and if this still has a negative impact on vascular function. Furthermore, it would also be of importance to determine if vascular ROS production is elevated and NO· function impaired at later time points after cerebral ischaemia (e.g. > 1 week) and determine if improving vascular function after stroke, improves long term neurological outcome.

As discussed, there is a growing body of evidence that implicates immune cells in a number of diseases affecting the cerebral circulation, including hypertension, hypercholesterolaemia and stroke (Brait *et al.*, 2010; Guzik *et al.*, 2007; Stokes *et al.*, 2007a; Stokes *et al.*, 2007b; Yilmaz *et al.*, 2006). While we have clearly demonstrated a detrimental role for Nox2-NADPH oxidase in the cerebral circulation, it is unclear if the source of Nox2-NADPH oxidase-derived ROS is vascular, immune cell or a combination of the two cell types. Thus, before we can therapeutically target Nox2-NADPH oxidase, further studies are needed to determine the cellular source of Nox2-NADPH oxidase-derived ROS.

Conclusion

In conclusion, the studies detailed in this thesis identify Nox2-NADPH oxidase as a deleterious source of ROS in the cerebral circulation. Specifically, Nox2-NADPH oxidase is the source of augmented ROS in cerebral arteries from: (1) males in response to angiotensin II; (2) during hypercholesterolaemia; and (3) following cerebral ischaemia-reperfusion. Furthermore, we show that the augmented Nox2-NADPH oxidase-derived

ROS production play a critical role in constrictor responses to angiotensin II in males and in reducing NO· bioavailability during hypercholesterolaemia and potentially following ischaemic stroke. The findings of the thesis highlight Nox2-NADPH oxidase as a potential therapeutic target for treating cerebrovascular dysfunction that is likely to be an underlying cause of cerebrovascular diseases such as stroke.

CHAPTER 8

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