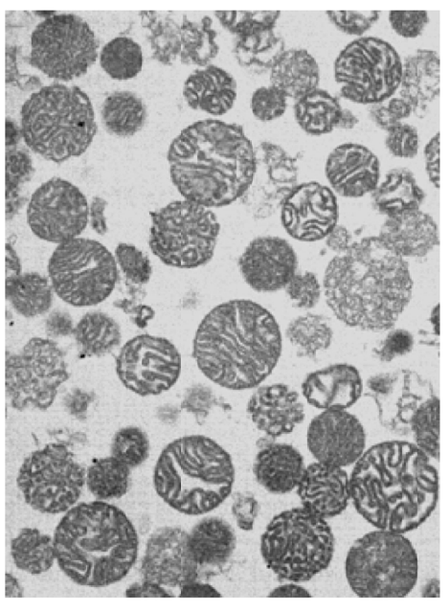
The role of mitochondria in cardioprotection

Heinen, A.

Publication date
2008
Document Version
Final published version

The Role of Mitochondria in Cardioprotection



André Heinen

The Role of Mitochondria in Cardioprotection

The Role of Mitochondria in Cardioprotection

André Heinen

The Role of Mitochondria in cardioprotection
André Heinen, Dissertation, University of Amsterdam, the Netherlands

This dissertation was prepared at the Department of Anesthesiology, Academic Medical Center (AMC), Amsterdam, the Netherlands.

This dissertation was funded in part by a by a MD-medical research trainee (AGIKO) grant (92003450) from the Netherlands Organisation for Health Research and Development (ZonMw, the Hague, the Netherlands).

Cover photo: Electron microscopy of isolated guinea pig cardiac mitochondria.
Thanks to Amadou K.S. Camara, Michelle M. Henry, Anna Fekete, and Janice Burke, Medical College of Wisconsin, Milwaukee, USA.

The Role of Mitochondria in Cardioprotection

Academisch Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties
ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op dinsdag 14 oktober 2008, te 12:00 uur

door

André Heinen
geboren te Mönchengladbach, Duitsland

Promotiecommissie:

promotores:	Prof. dr. M.W. Hollmann
	Prof. dr. W.S. Schlack
Co-promotores:	Dr. N.C. Hauck
	Dr. B. Preckel
Overige leden:	Prof. dr. S.G. de Hert
	Prof. dr. J.H. Ravesloot
	Prof. dr. M.C. Michel
	Prof. dr. R.J.A. Wanders
	Prof. dr. V. Thämer
	Prof. dr. D.F. Stowe

Faculteit der Geneeskunde

Contents

Chapter 1

Introduction

Ischemic heart disease, with its clinical consequences of acute myocardial infarction, sudden cardiac death, arrhythmias and heart failure is the leading cause of mortality in industrialized nations. (38; 55) The main cause of acute myocardial infarction is the occlusion of a coronary artery. A rapid reperfusion therapy is required to preserve myocardial tissue, because the extent of myocardial necrosis increases with growing duration of the ischemic period. (9; 48) Therapeutical options for the restoration of the coronary flow are angioplasty, thombolysis therapy, or surgical bypass grafting.
During the last decades, many efforts were made to develop new therapeutic strategies for the preservation of myocardial tissue. Important observations in basic cardiovascular research demonstrated the existence of endogenous protection mechanisms capable to increase the resistance of the myocardium against myocardial ischemia.

Cardioprotective mechanisms

Ischemic preconditioning

In 1986, Murry et al. demonstrated that short cycles of myocardial ischemia increase the resistance of the myocardium against the deleterious consequences of a subsequent prolonged period of myocardial ischemia. (39) This cardioprotective phenomenon is known as ischemic preconditioning. The protective effect of ischemic preconditioning lasts up to 3 hours after the preconditioning ischemia, and is called classic or early preconditioning. A "second window of protection" reappears 12-24 hours after the initial stimulus, lasts for about 2-3 days, and is known as late preconditioning. (7)
The cardioprotective effect of ischemic preconditioning has been demonstrated in all animal species studied, and in human myocardium. (28) Ischemic preconditioning may play an important role to reduce the extent of myocardial infarction in patients with a history of angina. (3)
Although much effort was taken to investigate the underlying mechanisms of ischemic preconditioning during the last two decades, the signaling pathway and the end-effector mechanisms of early and late preconditioning are still incompletely understood. Rapid posttranslational modifications of pre-existing proteins are involved in the signaling pathway of early preconditioning. It was proposed that the release of endogenous triggers causes activation of intracellular kinases including phosphatidylinositol 3-kinase (PI3-K), protein kinase B (Akt), mitogen-activated protein kinase (MAPK), and/or extracellular signal regulated kinase (Erk1/2). It is suggested that many of these signaling steps converge to the mitochondrion, with the consequence of alterations in mitochondrial bioenergetics.

(37) The synthesis of new cardioprotective proteins including NO synthase, cyclooxygenase-2 (COX-2), superoxide dismutase (SOD), and activation of stress-response transcription factors like NF-κB is involved in late preconditioning. (7)
Despite these differences, early and late preconditioning seem to share mechanisms in their respective signalling pathways during both the trigger phase and the ischemia-reperfusion phase. It has been shown that activation of protein kinase C (PKC) is involved in both early and late preconditioning. (1; 6) Furthermore, there is strong evidence that regulation of mitochondrial function due to mitochondrial ATP-sensitive and/or calcium-sensitive potassium (mK_{ATP} / mK_{Ca}) channel activation is a central element in preconditioning. (11; 34; 41; 50)

Pharmacological preconditioning

A cardioprotective effect similar to ischemic preconditioning can also be induced by transient pre-treatment with various pharmacological agents; this phenomenon is called pharmacological preconditioning. Most of these drugs act on receptors, enzymes, or ion channels that have been shown to be involved in the signal transduction pathway of ischemic preconditioning. For example, it is known that pharmacological activation of adenosine, β-adrenergic, muscarinergic, opioid or bradykinin receptors mimick the protective effect of ischemic preconditioning. (12; 49; 54; 60) Other drugs that are capable to initiate preconditioning are volatile anesthetics, noble gases, nitric oxide donors, angiotensin II receptor antagonists, angiotensin-converting enzyme inhibitors, and ethanol. (29; 36; 42; 43; 45; 57; 61) Furthermore, pharmacological activation of both mK_{ATP} channels and mK_{Ca} channels initiate a strong cardioprotective effect. (16; 41; 58)

Postconditioning

Recently, it has been demonstrated that the extent of myocardial cell death after an ischemic period can be reduced by conducting repetitive cycles of ischemia/reperfusion at the end of the prolonged phase of ischemia. This phenomenon is called ischemic postconditioning, and was first described by Zhao et al. (62) The authors have shown that the magnitude of myocardial infarct size reduction induced by postconditioning is comparable to that seen with ischemic preconditioning. (62) Furthermore, postconditioning improves left ventricular functional recovery in isolated mouse hearts. (26) The protective potency of postconditioning to reduce infarct size has been shown in a variety of experimental models and animal species including mouse, (22; 30) rat, (27; 53) rabbit, (2;

59) pig, (24), and dog. (62) There is also evidence that postconditioning protects the human myocardium. (33; 51)

Common pathways of preconditioning and postconditioning during early reperfusion
Recently, a common cardioprotective pathway has been proposed, in which the signalling mechanisms of both preconditioning and postconditioning converge during the reperfusion period. (21) It has been suggested that the PI3-K-Akt and the Erk1/2-MAPK pathways are phosphorylated in response to a preconditioning or postconditioning stimulus. The group of enzymes that confer cardioprotection when activated during the reperfusion period is called the reperfusion injury salvage kinase (RISK) pathway. The mechanism by which RISK pathway activation mediates cardioprotection is incompletely understood, but a mechanistic link from the RISK pathway via glycogen synthase kinase-3beta (GSK-3β) to inhibition of the mitochondrial permeability transition pore (mPTP) opening has been proposed. (25)

Role of mitochondria in cardioprotection

Mitochondria play an essential, multifactorial role within the cell. Alongside the synthesis of adenosine-triphosphate, mitochondria are involved in cellular Ca^{2+} homeostasis, (40) the regulation of apoptosis, (31) and the generation of reactive oxygen species. (10)
Mitochondria generate ATP primarily by oxidative phosphorylation through the chemiosmotic mechanism. (35) In this process, the reducing equivalents NADH and $FADH_2$, which are mainly products of the Krebs cycle and of oxidation of fatty acids, transfer electrons to the mitochondrial electron transport chain. The passage of electrons through the complexes I, III, and IV of the electron transport chain are coupled to a proton transport across the inner mitochondrial membrane. The resulting electrochemical gradient is the major component of the mitochondrial membrane potential ($\Delta\psi_m$), which serves as driving force for oxidative phosphorylation at complex V.
As described above, there is strong evidence that mitochondrial mechanisms are involved in the protective effects of preconditioning and postconditioning. Preconditioning can be initiated by pharmacological activation of mitochondrial mK_{ATP}. (16; 32; 46) Further support for a role of mitochondria to "trigger" preconditioning has been given by the finding that mK_{ATP} channel blockade abolishes ischemic preconditioning. (4; 23) Furthermore, another class of mitochondrial K^+ channels, the calcium sensitive potassium channel (mK_{Ca}), has been shown to mediate myocardial protection. (58) Pharmacological blockade of these channels abolishes the cardioprotective effect of ischemic

preconditioning, which suggests a central role of K^+ channel opening in preconditioning. (11; 50) It has been proposed that potassium influx into the mitochondrial matrix through activated potassium channels regulate mitochondrial bioenergetics. These alterations are capable to cause a release of free radicals (ROS), most likely derived from electron leak of the mitochondrial electron transport chain. The ROS have been proposed to be critically important for the activation of different kinases that act as triggers and/or mediators of cardioprotection. (5; 44; 52) The exact mechanism by which K^+ influx resulting from preconditioning-induced K^+ channel opening alters mitochondrial function, subsequently initiating myocardial protection, is still incompletely understood.

Mitochondria are not only important during the "trigger" phase of preconditioning, they are also involved in the putative end-effector mechanism of both preconditioning and postconditioning. A possible candidate as end-effector of cardioprotection against ischemia and reperfusion injury is the mPTP. (18; 20) The mPTP is a multiprotein complex formed at the contact sites between the inner and the outer mitochondrial membrane by several proteins including the adenine nucleotide translocase, the voltage-dependent anion channel, cyclophilin D, and hexokinase. (13) In the normal myocardium the inner mitochondrial membrane is relatively impermeable, as it must be to maintain a proton gradient (the driving force for oxidative phosphorylation) established by the mitochondrial respiration. Opening of the mPTP leads to a sudden increase in permeability of the inner mitochondrial membrane, loss of mitochondrial membrane potential (Ψ_m), swelling of the mitochondrial matrix with the consequence of outer mitochondrial membrane rupture and release of proteins into the cytosol (including pro-apoptotic cytochrome C). (47) The hypothesis that inhibition of mPTP is involved in the cardioprotective effect of preconditioning has been supported by Wang et al. (56) who demonstrated that nitric oxide (NO) induced preconditioning protects the heart against infarction via modulation of mPTP.

Opening of mPTP is triggered by cellular stress conditions including high [Ca^{2+}], oxidative stress, adenosine-triphosphate depletion, increased [P_i], and depolarization of the inner mitochondrial membrane, conditions that occur during ischemia and early reperfusion. (19) It has been shown that inhibition of mPTP opening by cyclosporine A prevents mitochondrial dysfunction, cytochrome C release and apoptosis in the rat heart. (8) In addition, Hausenloy et al. have demonstrated in isolated rat hearts that ischemic preconditioning as well as pharmacological preconditioning by mK_{ATP} channel activation confers cardioprotection by inhibition of mPTP at reperfusion. (20) The authors discussed three possible mechanisms by which preconditioning-induced altered mitochondrial function could be linked to inhibition of mPTP opening to protect the myocardium: a)

reduced mitochondrial Ca^{2+} overload due to a depolarization of Ψ_m (reduced driving force for Ca^{2+} inflow), b) improved mitochondrial adenosine-triphosphate levels, and c) reduced ROS production. All these alterations would prevent mPTP opening. (17; 19) Furthermore, another possible mechanism by which cardioprotective interventions could prevent mPTP opening is by activation of RISK pathway enzymes. The mechanism by which RISK pathway activation prevents mPTP opening is unclear, but it has been discussed that phosphorylation and inhibition of GSK-3β, a downstream target of the RISK pathway enzymes Akt and ERK1/2, is involved. (25)

AIMS OF THIS THESIS

The general aim of this thesis is to investigate the role of cardiac mitochondria in the signaling pathway of cardioprotection. The literature demonstrates that mitochondria and the regulation of mitochondrial processes are key components of cardioprotective interventions including preconditioning and postconditioning.

In **chapters 2 and 3** we study the regulation of mitochondrial bioenergetics by mK_{Ca} channels, whose activation has been shown to be important in preconditioning. For this, a sub-cellular approach of isolated cardiac mitochondria is used to investigate how pharmacological mK_{Ca} channel activation regulates mitochondrial respiration, membrane potential, and reactive oxygen production, and how these three parameters of mitochondrial function interrelate.

The cardioprotective potency of preconditioning has previously been demonstrated to be reduced in the senescent myocardium, probably due to some defects in mitochondrial K^+ channel function. In this context, we investigate in **chapter 4** whether the effects of pharmacological mK_{Ca} channel activation on mitochondrial respiration are age-dependent.

The investigation described in **chapters 5, 6, and 7** focusses on the cardioprotective properties of the noble gas helium. In these three studies the mechanisms of helium-induced preconditioning are investigated in *in vivo* experiments, in isolated cardiac mitochondria, and at the protein level. In **chapter 5** the involvement of mK_{Ca} channels in helium-induced preconditioning is investigated. We provide evidence that helium confers cardioprotection via regulation of mitochondrial function. Furthermore, we demonstrate that helium-induced preconditioning is abolished in the aged rat heart. In **chapter 6** we investigate the protective effects of helium in the diabetic heart, and in **chapter 7** we show that helium initiates late preconditioning.

In the last section of this thesis, we aim to investigate the mPTP in preconditioning and postconditioning. The study described in **Chapter 8** demonstrates that the cardioprotective effect of sevoflurane-induced postconditioning, which is abolished by hyperglycemia, can be restored by pharmacological blockade of the mitochondrial permeability transition pore. **Chapter 9** demonstrates in an impressive manner possible methodological pitfalls during the experimental testing of hypotheses. In this study, we aimed initially to investigate the role of mPTP inhibition in pharmacological preconditioning by morphine, but we failed to detect a protective effect of morphine at all. Therefore, this study resulted in a methodological investigation on the impact of experimental (substrate) conditions in isolated perfused heart experiments.

REFERENCES

1. **Ardehali H**. Signaling mechanisms in ischemic preconditioning: interaction of PKCepsilon and MitoK_{ATP} in the inner membrane of mitochondria. *Circ Res* 99: 798-800, 2006.
2. **Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D and Ovize M**. Postconditioning inhibits mitochondrial permeability transition. *Circulation* 111: 194-197, 2005.
3. **Arstall MA, Zhao YZ, Hornberger L, Kennedy SP, Buchholz RA, Osathanondh R and Kelly RA**. Human ventricular myocytes in vitro exhibit both early and delayed preconditioning responses to simulated ischemia. *J Mol Cell Cardiol* 30: 1019-1025, 1998.
4. **Auchampach JA, Grover GJ and Gross GJ**. Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res* 26: 1054-1062, 1992.
5. **Baines CP, Goto M and Downey JM**. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J Mol Cell Cardiol* 29: 207-216, 1997.
6. **Baxter GF, Goma FM and Yellon DM**. Involvement of protein kinase C in the delayed cytoprotection following sublethal ischaemia in rabbit myocardium. *Br J Pharmacol* 115: 222-224, 1995.
7. **Bolli R**. The late phase of preconditioning. *Circ Res* 87: 972-983, 2000.
8. **Borutaite V, Jekabsone A, Morkuniene R and Brown GC**. Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome c release and apoptosis induced by heart ischemia. *J Mol Cell Cardiol* 35: 357-366, 2003.
9. **Braunwald E**. The open-artery theory is alive and well--again. *N Engl J Med* 329: 1650-1652, 1993.
10. **Cadenas E and Davies KJ**. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 29: 222-230, 2000.
11. **Cao CM, Xia Q, Gao Q, Chen M and Wong TM**. Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning. *J Pharmacol Exp Ther* 312: 644-650, 2005.
12. **Cohen MV, Yang XM, Liu GS, Heusch G and Downey JM**. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K_{ATP} channels. *Circ Res* 89: 273-278, 2001.

13. **Crompton M**. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341 (Pt 2): 233-249, 1999.
14. **Das DK, Engelman RM and Maulik N**. Oxygen free radical signaling in ischemic preconditioning. *Ann N Y Acad Sci* 874: 49-65, 1999.
15. **Das DK, Maulik N, Sato M and Ray PS**. Reactive oxygen species function as second messenger during ischemic preconditioning of heart. *Mol Cell Biochem* 196: 59-67, 1999.
16. **Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA and Grover GJ**. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. *Circ Res* 81: 1072-1082, 1997.
17. **Griffiths EJ and Halestrap AP**. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *Biochem J* 307 (Pt 1): 93-98, 1995.
18. **Halestrap AP, Clarke SJ and Javadov SA**. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovasc Res* 61: 372-385, 2004.
19. **Halestrap AP, McStay GP and Clarke SJ**. The permeability transition pore complex: another view. *Biochimie* 84: 153-166, 2002.
20. **Hausenloy DJ, Maddock HL, Baxter GF and Yellon DM**. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res* 55: 534-543, 2002.
21. **Hausenloy DJ and Yellon DM**. Preconditioning and postconditioning: united at reperfusion. *Pharmacol Ther* 116: 173-191, 2007.
22. **Heusch G, Buchert A, Feldhaus S and Schulz R**. No loss of cardioprotection by postconditioning in connexin 43-deficient mice. *Basic Res Cardiol* 101: 354-356, 2006.
23. **Hide EJ and Thiemermann C**. Limitation of myocardial infarct size in the rabbit by ischaemic preconditioning is abolished by sodium 5-hydroxydecanoate. *Cardiovasc Res* 31: 941-946, 1996.
24. **Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E and Kremastinos DT**. Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. *Basic Res Cardiol* 101: 502-507, 2006.
25. **Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN and Sollott SJ**. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 113: 1535-1549, 2004.
26. **Kin H, Zatta AJ, Lofye MT, Amerson BS, Halkos ME, Kerendi F, Zhao ZQ, Guyton RA, Headrick JP and Vinten-Johansen J**. Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. *Cardiovasc Res* 67: 124-133, 2005.
27. **Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA and Vinten-Johansen J**. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiovasc Res* 62: 74-85, 2004.
28. **Kloner RA and Jennings RB**. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 2. *Circulation* 104: 3158-3167, 2001.
29. **Leesar MA, Stoddard MF, Dawn B, Jasti VG, Masden R and Bolli R**. Delayed preconditioning-mimetic action of nitroglycerin in patients undergoing coronary angioplasty. *Circulation* 103: 2935-2941, 2001.
30. **Lim SY, Davidson SM, Hausenloy DJ and Yellon DM**. Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc Res* 75: 530-535, 2007.
31. **Liu X, Kim CN, Yang J, Jemmerson R and Wang X**. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147-157, 1996.

32. **Liu Y, Sato T, O'Rourke B and Marban E**. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* 97: 2463-2469, 1998.
33. **Ma XJ, Zhang XH, Li CM and Luo M**. Effect of postconditioning on coronary blood flow velocity and endothelial function in patients with acute myocardial infarction. *Scand Cardiovasc J* 40: 327-333, 2006.
34. **Mei DA, Elliott GT and Gross GJ**. K_{ATP} channels mediate late preconditioning against infarction produced by monophosphoryl lipid A. *Am J Physiol* 271: H2723-H2729, 1996.
35. **Mitchell P and Moyle J**. Chemiosmotic hypothesis of oxidative phosphorylation. *Nature* 213: 137-139, 1967.
36. **Morris SD and Yellon DM**. Angiotensin-converting enzyme inhibitors potentiate preconditioning through bradykinin B2 receptor activation in human heart. *J Am Coll Cardiol* 29: 1599-1606, 1997.
37. **Murphy E and Steenbergen C**. Preconditioning: The mitochondrial connection. *Annu Rev Physiol* 69: 51-67, 2007.
38. **Murray CJ and Lopez AD**. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 349: 1498-1504, 1997.
39. **Murry CE, Jennings RB and Reimer KA**. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
40. **Nicholls D and Akerman K**. Mitochondrial calcium transport. *Biochim Biophys Acta* 683: 57-88, 1982.
41. **O'Rourke B**. Evidence for mitochondrial K^+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.
42. **Pagel PS, Krolikowski JG, Shim YH, Venkatapuram S, Kersten JR, Weihrauch D, Warltier DC and Pratt PF, Jr.** Noble gases without anesthetic properties protect myocardium against infarction by activating prosurvival signaling kinases and inhibiting mitochondrial permeability transition in vivo. *Anesth Analg* 105: 562-569, 2007.
43. **Pagel PS, Toller WG, Gross ER, Gare M, Kersten JR and Warltier DC**. K_{ATP} channels mediate the beneficial effects of chronic ethanol ingestion. *Am J Physiol Heart Circ Physiol* 279: H2574-H2579, 2000.
44. **Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV and Downey JM**. Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. *Circ Res* 87: 460-466, 2000.
45. **Sato M, Engelman RM, Otani H, Maulik N, Rousou JA, Flack JE, III, Deaton DW and Das DK**. Myocardial protection by preconditioning of heart with losartan, an angiotensin II type 1-receptor blocker: implication of bradykinin-dependent and bradykinin-independent mechanisms. *Circulation* 102: III346-III351, 2000.
46. **Sato T, Sasaki N, Seharaseyon J, O'Rourke B and Marban E**. Selective pharmacological agents implicate mitochondrial but not sarcolemmal K_{ATP} channels in ischemic cardioprotection. *Circulation* 101: 2418-2423, 2000.
47. **Scarlett JL and Murphy MP**. Release of apoptogenic proteins from the mitochondrial intermembrane space during the mitochondrial permeability transition. *FEBS Lett* 418: 282-286, 1997.
48. **Schomig A, Ndrepepa G and Kastrati A**. Late myocardial salvage: time to recognize its reality in the reperfusion therapy of acute myocardial infarction. *Eur Heart J* 27: 1900-1907, 2006.
49. **Schultz JE, Hsu AK and Gross GJ**. Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. *Circ Res* 78: 1100-1104, 1996.
50. **Shintani Y, Node K, Asanuma H, Sanada S, Takashima S, Asano Y, Liao Y, Fujita M, Hirata A, Shinozaki Y, Fukushima T, Nagamachi Y, Okuda H, Kim J, Tomoike H, Hori**

M and Kitakaze M. Opening of Ca^{2+}-activated K^+ channels is involved in ischemic preconditioning in canine hearts. *J Mol Cell Cardiol* 37: 1213-1218, 2004.

51. **Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, Aupetit JF, Bonnefoy E, Finet G, ndre-Fouet X and Ovize M**. Postconditioning the human heart. *Circulation* 112: 2143-2148, 2005.
52. **Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG and Jiang MT**. Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 290: H434-H440, 2006.
53. **Tsang A, Hausenloy DJ, Mocanu MM and Yellon DM**. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res* 95: 230-232, 2004.
54. **Tsuchida A, Liu Y, Liu GS, Cohen MV and Downey JM**. alpha 1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res* 75: 576-585, 1994.
55. **Tunstall-Pedoe H, Kuulasmaa K, Amouyel P, Arveiler D, Rajakangas AM and Pajak A**. Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation* 90: 583-612, 1994.
56. **Wang X, Yin C, Xi L and Kukreja RC**. Opening of Ca^{2+}-activated K^+ channels triggers early and delayed preconditioning against I/R injury independent of NOS in mice. *Am J Physiol Heart Circ Physiol* 287: H2070-H2077, 2004.
57. **Weber NC, Toma O, Wolter JI, Obal D, Mullenheim J, Preckel B and Schlack W**. The noble gas xenon induces pharmacological preconditioning in the rat heart in vivo via induction of PKC-epsilon and p38 MAPK. *Br J Pharmacol* 144: 123-132, 2005.
58. **Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A and O'Rourke B**. Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* 298: 1029-1033, 2002.
59. **Yang XM, Proctor JB, Cui L, Krieg T, Downey JM and Cohen MV**. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. *J Am Coll Cardiol* 44: 1103-1110, 2004.
60. **Yao Z and Gross GJ**. Acetylcholine mimics ischemic preconditioning via a glibenclamide-sensitive mechanism in dogs. *Am J Physiol* 264: H2221-H2225, 1993.
61. **Zaugg M, Lucchinetti E, Uecker M, Pasch T and Schaub MC**. Anaesthetics and cardiac preconditioning. Part I. Signalling and cytoprotective mechanisms. *Br J Anaesth* 91: 551-565, 2003.
62. **Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA and Vinten-Johansen J**. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 285: H579-H588, 2003.

Chapter 2

Mitochondrial Ca^{2+} -induced K^{+} influx increases respiration and enhances ROS production while maintaining membrane potential

André Heinen, Amadou K.S. Camara, Mohammed Aldakkak, Samhita S. Rhodes, Matthias L. Riess, and David F. Stowe

American Journal of Physiology - Cell Physiology
2007 Jan;292(1):C148-56

ABSTRACT

We recently showed a role for altered mitochondrial bioenergetics and reactive oxygen species (ROS) production in mitochondrial Ca^{2+} sensitive K^+ (mK_{Ca}) channel opening - induced preconditioning in isolated hearts. However, the underlying mitochondrial mechanism by which mK_{Ca} channel opening causes ROS production to trigger preconditioning is unknown. We hypothesized that submaximal mK^+ influx causes ROS production due to enhanced electron flow at a fully charged membrane potential (ψ_m). To test this we measured effects of NS1619, a putative mK_{Ca} channel opener, and valinomycin, a K^+ ionophore, on mitochondrial respiration, ψ_m, and ROS generation in guinea pig heart mitochondria. NS1619 (30 μM) increased state 2 and 4 respiration, respectively, by 5.2±0.9 and 7.3±0.9 nmol O_2/mg protein/min with NADH linked substrate pyruvate and by 7.5±1.4 and 11.6±2.9 nmol O_2/mg protein/min with $FADH_2$ linked substrate succinate (plus rotenone); these effects were abolished by the mK_{Ca} blocker paxilline. ψ_m was not decreased by 10-30 μM NS1619 with either substrate, but H_2O_2 release was increased by 44.8% (65.9±2.7% by 30 μM NS1619 vs. 21.1±3.8% for time-controls) with succinate + rotenone. In contrast, NS1619 did not increase H_2O_2 release with pyruvate. Similar results were found for lower concentrations of valinomycin. The increase in ROS production in succinate + rotenone supported mitochondria resulted from a fully maintained ψ_m despite increased respiration, a condition that is capable of allowing increased electron leak. We propose that mild matrix K^+ influx during states 2 and 4 increases mitochondrial respiration, while maintaining ψ_m this allows singlet electron uptake by O_2 and ROS generation.

INTRODUCTION

Mitochondrial bioenergetic effects of K^+ influx into the mitochondrial matrix is controversial. (13-15; 29) It has been proposed that opening of one class of K^+ channel in the inner mitochondrial membrane (IMM), the mitochondrial (m) ATP sensitive K^+ (mK_{ATP}) channel, leads to increased mitochondrial matrix volume, increased respiration, slightly reduced mitochondrial membrane potential (ψ_m), and matrix alkalinization. (22) On the other hand, Holmuhamedov et al. (17) demonstrated in isolated rat heart mitochondria that mK_{ATP} channel opening markedly depolarized ψ_m along with accelerated respiration, slowed ATP production, and increased matrix volume. Liu et al. (25) further supported the hypothesis that matrix K^+ influx stimulates mitochondrial respiration by reporting an increase in flavoprotein fluorescence in substrate depleted medium after treatment with the mK_{ATP} channel opener diazoxide.

Matrix K^+ influx may play an essential role in cardiac pharmacological preconditioning. In addition to the mK_{ATP} channel there is increasing evidence for a role of mitochondrial Ca^{2+} sensitive potassium (mK_{Ca}) channel opening in cardioprotection. Xu et al. (42) found big (B) conductance (mBK_{Ca}) channels in the IMM of guinea pig ventricular cells and demonstrated their protective potency against ischemia reperfusion injury. Recently, Cao et al. (5) demonstrated that pharmacological preconditioning was initiated by the mBK_{Ca} channel activator 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoro-methyl)-2H-benzimidazol-2-one (NS1619) in isolated rat hearts. Blockade of mBK_{Ca} channels abolished the reduction of infarct size caused by ischemic preconditioning (IPC), which suggested a key role of mBK_{Ca} channel opening in ischemic preconditioning. (35) Opening of mBK_{Ca} channels by NS1619 also triggered delayed preconditioning against ischemia reperfusion injury in mice. (41) Sato et al. (34) confirmed the protective effect of NS1619 in isolated cardiac myocytes, where pretreatment with NS1619 reduced mitochondrial Ca^{2+} overload -induced cell death, and increased flavoprotein fluorescence. These authors suggested this increase in flavoprotein fluorescence, indicating a more oxidized redox state, is caused by enhanced mitochondrial respiration as a consequence of K^+ influx into the mitochondrial matrix.

We reported recently (37) that preconditioning by NS1619 aids in preserving the mitochondrial redox state, lowers reactive oxygen species (ROS) production, and reduces mitochondrial Ca^{2+} overload during both ischemia and reperfusion in isolated guinea pig hearts. This protection was blocked by bracketing NS1619 treatment with either the BK_{Ca} channel blocker paxilline or the superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic

acid) porphyrin chloride (MnTBAP). This finding supported a hypothesis that ROS play a key role in triggering cardiac preconditioning and that this effect is modulated by matrix K^+ influx and subsequent changes in mitochondrial bioenergetics. Indeed a role for ROS in initiating ischemic and pharmacological preconditioning by mK_{ATP} channel opening is now strongly supported by individual studies (12; 19; 31; 40), and is summarized in recent reviews.(16; 38; 43)

It is unknown how mBK_{Ca} channel opening alters mitochondrial function, and what impact such alterations have on mitochondrial ROS generation. Because a key initiating trigger in cardiac preconditioning may be K^+ influx –related ROS generation, we investigated effects of drug -induced mBK_{Ca} channel opening, and valinomycin –induced K^+ influx on respiration, ψ_m, and H_2O_2 release rate in cardiac isolated mitochondria. Our aim was to determine the respiratory conditions by which matrix K^+ influx, specifically via the mBK_{Ca} channel, can induce an increase in mitochondrial ROS generation, an essential condition required for initiation of cardiac preconditioning.

MATERIALS AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health No. 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committee (Medical College of Wisconsin, Milwaukee, Wisconsin).

Mitochondrial isolation

Heart mitochondria were isolated from ketamine-anesthetized guinea pigs (250-300 g) of either sex by differential centrifugation as described previously (33) with moderate modifications. Briefly, ventricles were excised, placed in an isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mmol/L KH_2PO_4, 5 mM 3-(n-morpholino) propranesulfonic acid (MOPS), 1 mM EGTA, 0.1% bovine serum albumin (BSA), pH 7.15 adjusted with KOH), and minced into 1 mm^3 pieces. The suspension was homogenized for 15 s in 2.5 mL isolation buffer containing 5 U/mL protease (*bacillus licheniformis*), and for another 15 s after addition of 17 mL isolation buffer. The suspension was centrifuged at 8000*g* for 10 min; the pellet was resuspended in 25 mL isolation buffer and centrifuged at 750*g* for 10 min, the supernatant was centrifuged at 8000*g* for 10 min, and the final pellet was suspended in 0.5 mL isolation buffer and kept on ice. The protein content was determined by the Bradford method. (2) All isolation procedures were conducted at 4˚C. All

experiments were conducted at a maintained temperature of 27˚C rather than at 37˚C via a circulatory water system to allow time to assess drug effects at a lower respiratory rate.

Mitochondrial O_2 consumption

Oxygen consumption was measured polarographically using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.25 mg protein/mL) were suspended in respiration buffer containing 130 mM KCl, 5 mM K_2HPO_4, 20 mM MOPS, 2.5 mM EGTA, 1 μM $Na_4P_2O_7$, 0.1% BSA, pH 7.15 adjusted with KOH. Buffer [Ca^{2+}] was less than 100 nM as assessed by the fluorescence dye indo 1. Respiration was initiated by administration of either pyruvate (P, 10 mM), or succinate (S, 10 mM) + rotenone (R, 10 μM). State 3 respiration was determined after addition of 125 nmoles (or 5 μl of 250 μM) ADP. The respiratory control index (RCI) was calculated as the state 3/state 4 ratio. Results were expressed as absolute changes from control in nmol O_2/mg/min or as percent of control.

Mitochondrial ψ_m

Mitochondrial membrane potential (ψ_m) was monitored during states 2-4 respiration with substrates pyruvate or succinate + rotenone in a cuvette-based spectrophotometer (QM-8, Photon Technology International, PTI) operating at excitation and emission wavelengths of 503 nm and 527 nm, respectively, in the presence of the fluorescence dye rhodamine 123 (50 nM). Mitochondria (0.5 mg/mL) were suspended in respiration buffer. ψ_m was expressed as the percentage of rhodamine 123 fluorescence in the presence of fully coupled mitochondria relative to the fluorescence after addition of 4 μM carbonyl-cyanide-m-chlorophenylhydrazenone (CCCP), a mitochondrial uncoupler.

Mitochondrial H_2O_2 release

Rates of mitochondrial H_2O_2 –release were measured spectrophotometrically (QM-8, PTI) with pyruvate or succinate + rotenone during states 2-4 respiration using the fluorescence dye amplex red (25 μM; Molecular Probes) in the presence of 0.1 U/mL horseradish peroxidase. Excitation and emission wavelengths were set to 530 nm and 583 nm, respectively. Mitochondria (0.5 mg/mL) were suspended in respiration buffer, and rates of H_2O_2 -release were expressed as percentage of baseline H_2O_2 –release (after substrate addition). Baseline H_2O_2 levels were calibrated from a mean of 3 standard curves of photon counts over a range of 10-200 nM H_2O_2 (added to assay medium in the presence of

reactants amplex red and horseradish peroxidase); each regression was linear (R >0.99). Time -controls received 0.3% dimethyl sulfoxide (DMSO), the dye vehicle.

Chemicals and reagents

Rhodamine 123, amplex red, and indo 1 were purchased from Molecular Probes (Eugene, OR) and KCl from EMD Chemicals (Gibbstown, NJ); all other chemicals were purchased from Sigma Chemical Co. NS1619, paxilline, and amplex red were dissolved in DMSO before they were added to the experimental buffer.

Statistical analyses

All data were analyzed using customized software developed in Matlab® (Mathworks, Nattick, MA). Group data were compared by analysis of variance. If *F* values (P <0.05) were significant, post hoc comparisons of means tests (Student-Newman-Keuls) were considered statistically significant when $P < 0.05$ (two-tailed). Data are presented as means ±SEM.

RESULTS

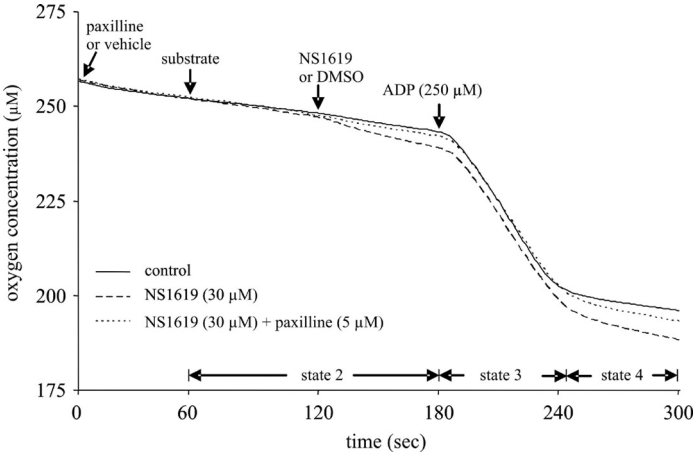
Mitochondrial respiration

Representative traces of respiration (Figure 1) and average values for control groups (Table 1, panel A) showed a high functional quality of mitochondria after the isolation procedure. In particular, the RCI of 11.3±1.0 for complex I substrate pyruvate, and 2.6±0.1 for complex II substrate succinate + rotenone, demonstrated a strong coupling between mitochondrial respiration and oxidative phosphorylation.

Table 1, panel B summarizes concentration-dependent effects of NS1619 on mitochondrial respiration for both substrates in normal K^+ buffer. NS1619 dose -dependently increased O_2 consumption during the resting states (state 2 and 4). With pyruvate as substrate, 10, 20, and 30 μM NS1619 had no effect on state 3 respiration; however, 50 μM NS1619 markedly decreased state 3 respiration. With succinate + rotenone as substrate, each concentration of NS1619 decreased state 3 respiration. Each concentration of NS1619 slightly reduced RCI under both substrate conditions, indicating mild uncoupling.

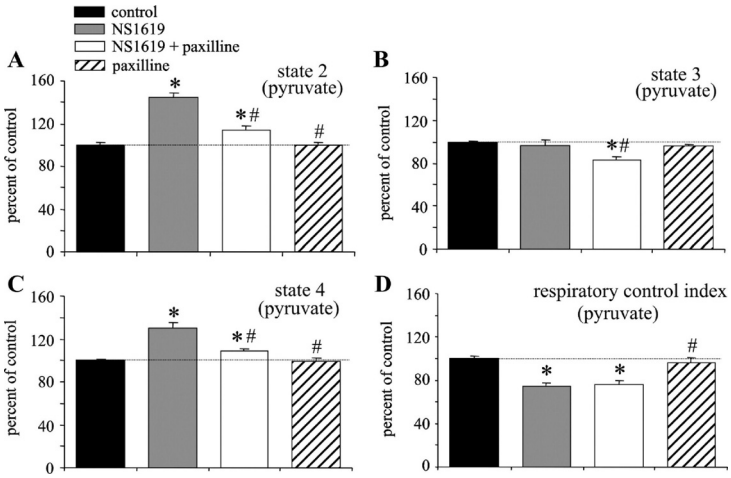
To test if these effects of NS1619 were due to mBK$_{Ca}$ channel opening, we added 5 μM paxilline in the absence or presence of 30 μM NS1619 (Figures 2 and 3).

Figure 1: Representative traces of mitochondrial respiration experiments.



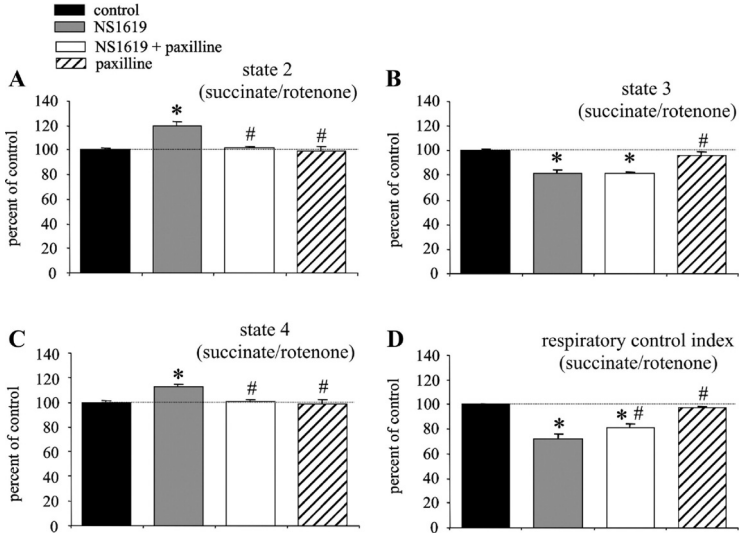
State 2 respiration was initiated by addition of 10 mM pyruvate; state 3 respiration was initiated by addition of 250 µM ADP. NS1619 or its vehicle DMSO (0.3 %) were administered at about 120 sec (treatment effects on state 2 were measured beginning at 120 sec) in the presence or absence of 5 µM paxilline.

Figure 2:



Summarized data for the effects of 30 µM NS1619 on mitochondrial respiration and the antagonist effects of mK_{Ca} channels by 5 µM paxilline in the presence of complex I substrate pyruvate (P, 10 mM). **P* < 0.05 vs. control; #*P* < 0.05 vs. NS1619.

Figure 3:



Summarized data for the effects of 30 µM NS1619 on mitochondrial respiration and the antagonist effects of mK_{Ca} channels by 5 µM paxilline in the presence of complex II substrate succinate (S, 10 mM) plus complex I blocker rotenone (R, 10 µmol/L). *P < 0.05 vs. control; #P < 0.05 vs. NS1619.

Paxilline alone had no effect on state 2 respiration indicating mBK_{Ca} channels were closed under the experimental conditions. Pre-administration of paxilline (for protocol see Figure 1) attenuated the 30 µM NS1619-induced increase in state 2 (14.6±4.3% vs. 46.9±7.2%) and state 4 (9.8±1.7% vs. 31.3±5.7%) respiration with pyruvate as substrate (figure 2) and abolished the NS1619-induced increase of state 2 (5.3±2.0% vs. 21.4±4.2%) and state 4 (1.5±1.6% vs. 13.5±1.9%) respiration with succinate + rotenone as substrate (figure 3). State 3 respiration was not affected by 30 µM NS1619, but prior administration of paxilline before NS1619 decreased state 3 respiration by 17.4±3.1% vs. control with pyruvate as substrate (figure 2). With succinate + rotenone as substrate, paxilline had no effect on the NS1619-induced decrease in state 3 respiration.

The decrease in RCI with pyruvate was not affected by prior administration of paxilline, but was blunted (19.6±3.4% vs. 29.0±3.9%) with succinate + rotenone. These data demonstrate that NS1619 increases mitochondrial state 2 and 4 respiration, but not state 3 respiration, by activating mBK_{Ca} channels.

Table 1:

A control respiration			state 2	state 3	state 4	RCI	
				(nmol O_2/mg/min)		(state 3/state 4)	n
pyruvate	(P)	control	17.3±0.9	190.2±14.1	17.2±0.8	11.3±1.0	9
succinate + rotenone	(S/R)	control	59.0±3.2	151.3±9.7	58.8±3.0	2.6±0.1	9

B changes from control			state 2	state 3	state 4	RCI	
				(nmol O_2/mg/min)		(state 3/state 4)	n
pyruvate	(P)	NS 10	2.1±1.1	2.5±7.3	1.4±0.7	-0.8±0.3	8
		NS 20	4.5±0.6*	-2.0±6.9	4.0±0.8	-2.3±0.3*	8
		NS 30	7.3±0.9*	-2.9±9.2	5.2±0.9*	-2.8±0.4*	8
		NS 50	33.0±2.2*	-37.7±6.2*	23.8±2.4*	-7.9±0.9*	8
succinate + rotenone	(S/R)	NS 10	-0.8±1.6	-19.8±3.2*	-1.8±1.9	-0.3±0.1*	8
		NS 20	5.3±1.5	-25.0±3.6*	0.8±1.9	-0.5±0.1*	8
		NS 30	11.6±2.9*	-26.7±6.8*	7.5±1.4*	-0.7±0.1*	8
		NS 50	23.0±5.5*	-65.7±5.2*	----.	----.	8

Oxygen consumption of mitochondria respiring in pyruvate or succinate + rotenone in the absence or presence of NS1619. Note the concentration dependent increases during states 2 and 4 and the decrease in state 3 only with 50 µM NS1619. RCI = respiratory control ratio; *P<0.05 vs. control; n = number of hearts

Mitochondrial ψ_m

We measured fluorescence of the membrane potential sensitive dye rhodamine 123 to examine the effects of NS1619 and valinomycin on ψ_m. Data are shown both as raw photon counts/s (Table 2) and as % of maximal depolarization by CCCP (Figure 4). Administration of 30 µM NS1619 had no effect on ψ_m with complex I substrate pyruvate (0.7±0.2% vs. 0% baseline) or complex II substrate succinate + rotenone (-0.4±0.3% vs. 0% baseline) (Figure 4, panels A and B). A significant depolarization of ψ_m was detected after administration of 50 µM NS1619, and this effect was substrate-independent (2.7±0.4% vs. baseline for pyruvate, 2.7±0.6% vs. baseline for succinate + rotenone). Note that state 2 and 4 respiratory rates were approximately three-fold higher with succinate + rotenone than with

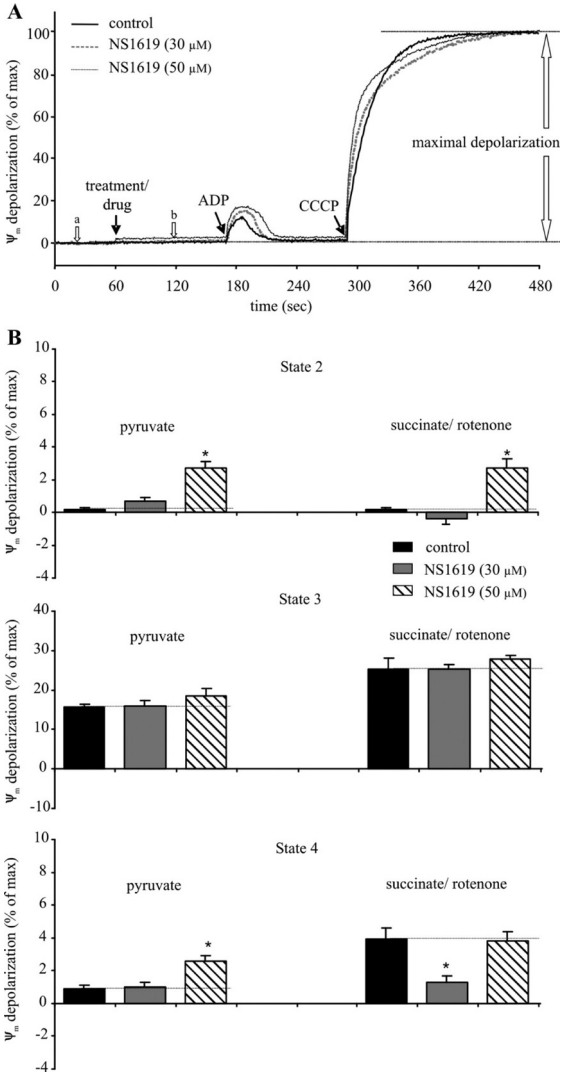
pyruvate (Table 1). The large ADP -induced (state 3) peak depolarization was unaffected by 30 or 50 µM NS1619. During state 4 respiration with pyruvate, effects on ψ_m were similar to those of state 2, i.e., depolarization only at 50 µM NS1619. With succinate + rotenone, 30 µM NS1619 promoted a hyperpolarization and 50 µM NS1619 caused no change in ψ_m relative to the controls. These data indicate that during state 2 and 4, NS1619 maintains (or increases) ψ_m, which favors generation of $O_2^{\bullet-}$. The extent and duration of ADP -induced depolarization was slightly enhanced in the NS1619 (50 µM) group compared to the time-control group; however, these effects did not reach statistical significance (data not shown).

Table 1:

pyruvate	n	state	Photons 1000/s	Succinate/ Rotenone	n	state	Photons 1000/s
	6	2	0.8 ±0.0		5	2	0.4 ±0.3
control	6	3	41.9 ±3.2	control	5	3	71.3 ±19.0
	6	4	3.1 ±0.5		4	4	10.0 ±1.7
	5	2	1.1 ±0.3		5	2	0.3 ±0.4
NS1619 30 µM	5	3	44.7 ±4.9	NS 30 µM	5	3	66.9 ±4.0
	5	4	1.7 ±1.1		4	4	1.6 ±0.8*
	5	2	10.3 ±1.6*		5	2	10.0 ±1.6*
NS1619 50 µM	5	3	57.1 ±5.7	NS 50 µM	5	3	62.4 ±3.3
	5	4	12.7 ±3.2*		5	4	9.9 ±1.6

Photons/s indicative of changes in membrane potential (ψ_m) assessed by the fluorescence probe rhodamine 123. Note that with pyruvate as substrate only 50 µM NS1619 increased state 2 and state 4 ψ_m; with succinate and rotenone 30 µM NS1619 decreased state 4 ψ_m and 50 µM NS119 increased state ψ_m. Fig. 4 shows that the data normalized to % maximal depolarization of ψ_m by CCCP furnished qualitatively and statistically the same results. *P<0.05 vs. control; n = number of hearts

Figure 4:

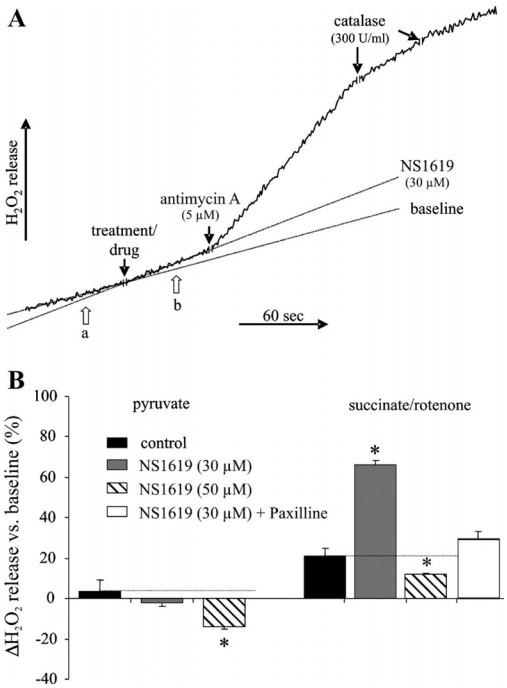


Effects of NS1619 on ψ_m. Panel A: Representative traces for ψ_m measurements with pyruvate as substrate. NS1619 (30 or 50 µM) or its vehicle DMSO (0.3%) were added. To verify the functional integrity of mitochondria and ψ_m, 250 µM ADP was added as indicated. Maximal depolarization was measured after addition of 4 µM CCCP, a mitochondrial uncoupler (arrow a: baseline = 0% depolarization; arrow b: treatment effect). Panel B: Summarized data for ψ_m. All treatment effects (b) are compared to baseline (a) of the same experiment. (*P < 0.05 vs. control; n = 8 for S/R control, n = 5 for all other groups)

Mitochondrial ROS generation

Mitochondrial H_2O_2 release rate was increased by 30 μM NS1619 (Figure 5, panel A); antimycin A (complex III inhibitor) caused a greater increase in H_2O_2 and catalase (converts H_2O_2 to H_2O) inhibited its release. It is well known that mitochondria generate ROS, initiated in the form of superoxide radical ($O_2^{\bullet-}$), by direct electron transfer to O_2 (electron leak), which is converted by superoxide dismutase to H_2O_2. (39) In the presence of complex I substrate pyruvate, activation of mBK_{Ca} channels by 30 μM NS1619 had no effect on the state 2 mitochondrial H_2O_2 release rate compared to the DMSO treated time-control group (-2.2±1.9% vs. 3.5±5.6%) (Figure 5, panel B).

Figure 5:



Mitochondrial H_2O_2 release rate measurements. Panel A: Representative trace for NS1619 (30 μM) - induced increase in cumulative H_2O_2 release with succinate + rotenone (S/R) as substrate. Maximal ROS production was stimulated in some experiments by addition of 5 μM complex III blocker antimycin A. Catalase (300 U/mL) was added to confirm H_2O_2 production. (arrow a: baseline; arrow b: treatment effect) Panel B: Summarized data for H_2O_2 release rate. All treatment effects are compared to baseline of the same experiment. A 10% change represents a change in H_2O_2 release rate of approximately 1.5 pmol•mg^{-1}• min^{-1} protein. (*P < 0.05 vs. control; n = 5 for each group)

In contrast, with succinate + rotenone, which stimulated at least a three-fold faster rate of respiration and potential for electron leak, 30 μM NS1619 increased the rate of H_2O_2 release by 44.8% (65.9±2.7% for NS1619 vs. 21.1±3.8% for time-controls). Furthermore, the large 30 μM NS1619-induced increase in H_2O_2 release rate was sensitive to mBK_{Ca} channel blockade; pretreatment with 5 μM paxilline (n = 5) blunted the effect of NS1619 to 29.4±4.9% vs. 65.9±2.7% for NS1619 alone (Figure 5B).

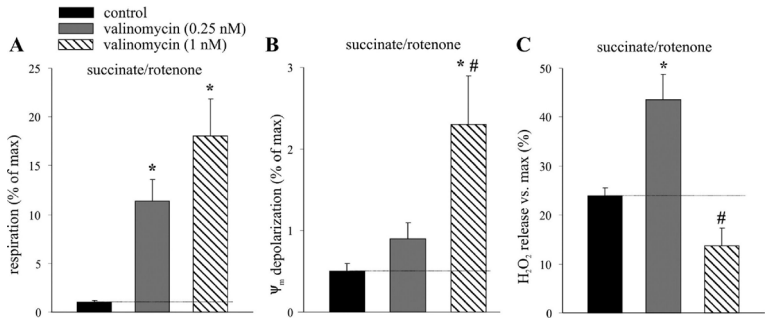
Interestingly, the high concentration of NS1619 (50 μM) did not enhance H_2O_2 release rate; in contrast, the H_2O_2 release rate was reduced (-13.9±1.1% vs. 3.5±5.6%) for pyruvate, and slightly (not significant) diminished with succinate + rotenone (11.7±0.6% vs. 21±3.8%) compared to time-controls. This was accompanied by conditions of much faster respiratory rates, but a 2.5% average depolarization of ψ_m under both substrate conditions. During state 4 respiration there was no change in H_2O_2 release rates by 30 or 50 μM NS1619 with pyruvate (data not displayed); with succinate + rotenone during state 4, 30 μM NS1619 significantly increased the H_2O_2 release rate (24.3±2.5%) over the no drug control, whereas 50 μM NS1619 had no effect (-3.5±3.3%) (n = 4; $P < 0.05$ (data not plotted).

K^+ ionophore valinomycin on respiration, ψ_m, and ROS release rate

To support the hypothesis that matrix K^+ influx causes ROS generation when respiration is increased but ψ_m is maintained, we conducted a series of experiments using the K^+ ionophore valinomycin with succinate + rotenone in normal K^+ buffer. The experimental protocol was the same as for NS1619 experiments. Figure 6 shows that the low concentration of valinomycin (0.25 nM) increased the state 2 respiration rate by 10.3±2.8% over the control rate (panel A), had no significant effect on ψ_m (panel B), and increased mitochondrial H_2O_2 release rate by 19.6% over the control rate (panel C). In comparison, 1 nM valinomycin increased state 2 respiration rate by 16±3.9%, depolarized ψ_m significantly over control (panel B), and did not alter the H_2O_2 release rate (panel C).

Similar to the results for lower concentrations of NS1619, this data shows that at a low concentration, valinomycin can also enhance ROS release when respiration is increased but ψ_m remains fully polarized.

Figure 6:



Effects of the K^+ ionophore valinomycin on respiration (*A*), ψ_m (*B*), and H_2O_2 release (*C*) with succinate + rotenone as substrate (state 2 respiration). All treatment effects are compared with baseline of the same experiment. Values are means ± SE; *n* = 5 for each group. **P* < 0.05 vs. control. #*P* < 0.05 vs. 0.25 nM valinomycin.

DISCUSSION

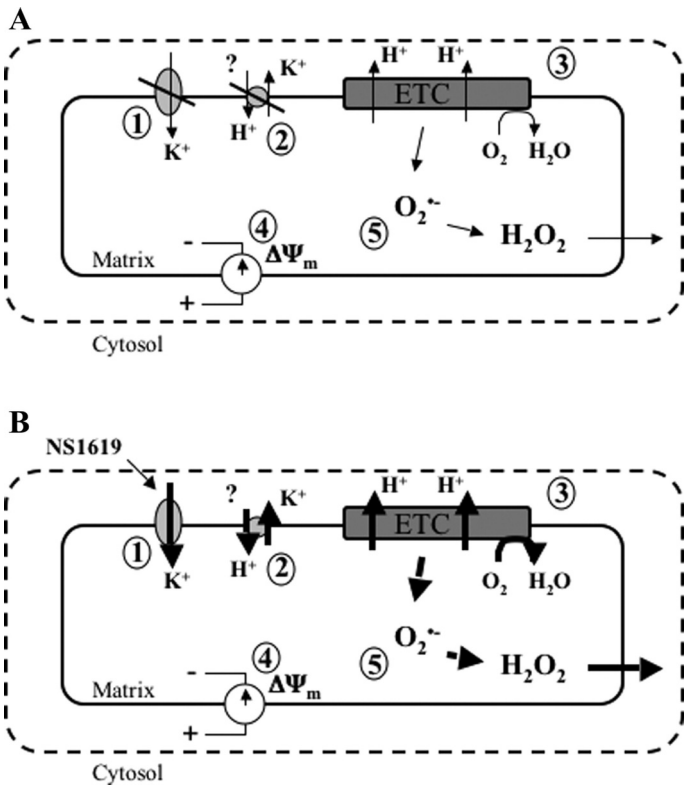
The major conclusions of this study are that a) limited matrix K^+ influx by putative mBK$_{Ca}$ channel activation by NS1619 and by the K^+ ionophore valinomycin increased stepwise the rate of mitochondrial respiration during the resting states 2 and 4, b) the resting state effects of NS1619 were blocked by paxilline; c) NS1619 depolarized ψ_m only at high concentrations (50 μM); and d) 0.25 nM valinomycin, and 30 μM NS1619, markedly increased mitochondrial ROS production in the presence of succinate + rotenone due to maintenance of a high resting ψ_m in the face of markedly enhanced respiration. From our data we offer a possibility by which ROS can be generated by submaximal K^+ induced H^+ leak into the mitochondrial matrix. Fig. 7 depicts a schematic representation of the putative pathway from mBK$_{Ca}$ channel opening to ROS generation.

Mitochondrial BK$_{Ca}$ channel opening alters mitochondrial respiration

Our finding that mBK$_{Ca}$ channel activation increased mitochondrial state 2 (and 4) respiration, an effect that was blocked by paxilline, is in agreement with the findings of Sato et al. (34), who demonstrated that NS1619 increased flavoprotein oxidation in a dose dependent manner in substrate depleted ventricular myocytes and that these effects were sensitive to the BK$_{Ca}$ channel blocker paxilline. We also demonstrated that paxilline alone had no effect on mitochondrial respiration indicating that mBK$_{Ca}$ channels were closed

under our experimental condition of low buffer [Ca^{2+}]. Several recent studies have demonstrated a stimulating effect on respiration of matrix K^+ influx through K_{ATP} channels. (8; 17; 27) O'Rourke (29) suggested that mitochondrial K^+ channels function as energy (stored as the proton gradient, $\Delta\mu H$) dissipating channels by expending $\Delta\mu H$, in part to eject K^+ that enters the matrix via activated K^+ channels via an electroneutral K^+/H^+ exchanger. This decrease in $\Delta\mu H$ would stimulate respiration to compensate for a net proton leak with the consequence of a maintained ψ_m.

Figure 7:



Proposed effect of submaximal matrix K^+ influx with mBK_{Ca} channel opening (1) on proton leak (2), proton ejection and respiration (3), membrane potential, ψ_m, (4), and generation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (5). **A**. mBK_{Ca} channel closed; **B**. mBK_{Ca} channel open. The net effect of mBK_{Ca} channel opening would be to accelerate electron flow without a change in ψ_m due to support by proton leak; the maintained ψ_m and higher electron flow would lead to more ROS generation. There are other promoters of proton leak that are not shown. ETC = electron chain transport.

Our finding that 10-30 μM NS1619 stimulated respiration, but did not depolarize ψ_m, validates this theory. Furthermore, we detected an inhibition of respiration under ADP stimulated state 3 conditions. Because this inhibitory effect was insensitive to paxilline, this indicates a mBK_{Ca} channel independent effect of NS1619. A similar effect of NS1619 was described by Debska et al. (7), who showed that NS1619 reduced the O_2 consumption rate after maximal stimulation of electron flow by the mitochondrial uncoupler dinitrophenol in glioma cells. In addition, Kowaltowski et al. (22) demonstrated a decrease in maximal respiratory rates in uncoupled mitochondria by the K_{ATP} channel openers diazoxide and pinacidil; but this effect was apparently unrelated to mitochondrial K_{ATP} channel activity because the decrease in respiration was also observed in K^+ free medium.

Most recently, Costa et al. (6) examined the effects of matrix K^+ influx in isolated rat heart mitochondria respiring on succinate + rotenone with oligomycin added to inhibit ATP synthesis at complex V and thus matrix proton influx, which prevents a depolarization of ψ_m. Under these conditions they demonstrated that matrix K^+ uptake is increased by either mK_{ATP} channel opening or by valinomycin and that this results in matrix swelling, mild uncoupling, and matrix alkalinization; these effects were blocked by ATP, glibenclamide and 5 hydroxydecanoate (5-HD). They proposed that the increase in matrix volume stimulates K^+/H^+ antiport activity, which tends to mitigate the matrix expansion indirectly by extrusion of K^+ for H^+. Finally, our group has reported that, similar to NS1619, the K_{ATP} channel openers, diazoxide and pinacidil, decreased ADP stimulated (state 3) respiration, but increased respiration in the presence of oligomycin (state 4) [preliminary observations (32)]; the latter, but not the former state condition was blocked by the K_{ATP} channel inhibitors 5-HD and glibenclamide.

Sub-threshold mBK_{Ca} channel opening does not alter ψ_m

In these experiments we demonstrated that mK_{Ca} channel activation by 10-30 μM NS1619 had no effect on ψ_m. We observed a slight depolarization only at 50 μM NS1619, a concentration that is higher than used in our prior study to examine effects of mBK_{Ca} channel opening on *ex situ* mitochondrial function or to initiate preconditioning (32). However, Sato et al. (34) observed that 30 μM NS1619 reduced ψ_m by 11% in ventricular myocytes; the difference in their study compared to ours may be due to their use of substrate depleted intact myocytes.

The effect of matrix K^+ influx through mK_{ATP} channels on ψ_m remains controversial. This may be due in part to the concentrations of drugs used to open these channels. Although a depolarizing effect of ψ_m by activating mK_{ATP} channels has been reported (17; 18; 28),

Kowaltowski et al. (22) argued against those findings because they believed the concentrations of K_{ATP} channel opener were too high and that the observed depolarization was not due to a K_{ATP} channel specific effect. Our data for the mBK_{Ca} channel supports their observations on the mK_{ATP} channel. They estimated that pharmacological concentrations of a mK_{ATP} channel opener would reduce ψ_m by only 1-2 mV. Our data also support the general hypotheses by O'Rourke (29) and Kowaltowski et al. (22) that matrix K^+ influx stimulates respiration by a reduced $\Delta\mu H$ due to exchange of K^+ for H^+ via the K^+/H^+ exchanger. We propose that mBK_{Ca} channel opening and matrix K^+ influx leads to H^+ influx (leak) by K^+/H^+ exchange, that in turn enhances matrix H^+ pumping and increases electron flow, and in resting states, prevents depolarization of ψ_m because protons cannot enter the matrix via complex V ATP synthase. The bioenergetic consequence of mK_{Ca} channel opening would be accelerated cycling of K^+ ions between the matrix and the intermembrane space (i.e. matrix K^+ inflow through mK_{Ca} channel, K^+ extrusion via K^+/H^+ exchanger) and an increase in mitochondrial respiration. Pronounced depolarization of ψ_m would occur only when the compensatory mechanism is exhausted, for example if matrix K^+ influx exceeds K^+/H^+ exchange capability, or under state 3 conditions. The interrelationship between mitochondrial ROS generation and basal and inducible H^+ leak by uncoupling proteins and the AMP/ANT pathway has been expertly reviewed recently in an article by Brookes. (3)

Mitochondrial BK_{Ca} channel opening enhances mitochondrial ROS generation

Our data demonstrate that opening of mBK_{Ca} channels by NS1619 (10-30 μM) enables increased ROS generation in isolated heart mitochondria during resting state conditions with complex II substrate succinate + rotenone. This increase in ROS generation was sensitive to paxilline, which indicates a mK_{Ca} channel opening -induced effect. The consequence of matrix K^+ influx by mK_{ATP} channel opening on mitochondrial ROS generation has been frequently discussed and remains quite controversial. Several laboratories demonstrated that activation of K_{ATP} channels causes increased ROS production (12; 13), whereas Ferranti et al. (11) reported that K_{ATP} channel opening decreases ROS generation.

The larger increase in H_2O_2 release rate in our study was induced by 30 μM NS1619, a concentration that also accelerated mitochondrial respiration, but had no effect on ψ_m. It appears unlikely that accelerated respiration (i.e. electron flow) alone can cause ROS generation to increase, because 50 μM NS1619, which further stimulated respiration, had no effect on the rate of mitochondrial H_2O_2 release. It is well accepted that mitochondrial

ROS production is strongly dependent on ψ_m; ROS generation can decrease rapidly with even a slight depolarization of ψ_m. (20; 21; 36) We suggest that 50 μM NS1619 leads to net K^+ influx not replaced by H^+ due to saturated K^+/H^+ exchange; this would cause a slight ψ_m depolarizing effect and more effective electron transfer with less chance for electron leak and ROS generation. Our finding that a low concentration (0.25 nM) of the K^+ ionophore valinomycin also increased ROS generation while ψ_m was not depolarized supports the hypothesis that submaximal matrix K^+ influx can increase ROS production. The higher concentration of valinomycin (1 nM), which depolarized ψ_m in a manner similar to that of 50 μM NS1619, also did not enhance the rate of H_2O_2 release. Brookes et al. (4) observed that 0.01 -1 nM valinomycin was sufficient to fully establish ψ_m, whereas 3 nM caused secondary proton flux with depolarization of ψ_m.

Despite our plausible mechanism for increased ROS generation by induced matrix K^+ influx, it is difficult to explain why no increase in ROS generation was detected by 30 μM NS1619 with 10 mM of the NADH linked substrate pyruvate. The net effect of 30 μM NS1619 on enhancing state 2 respiration was higher (11.6±2.9 nmol O_2/mg/min) with succinate + rotenone than with pyruvate (7.3±0.9 nmol O_2/mg/min). It is possible that the smaller increase in respiration and electron flow with pyruvate induces less ROS release, which may not be detectable by our fluorescence methods. There are now several lines of evidence that diazoxide, a putative K_{ATP} sensitive channel opener, can inhibit complex II and retard succinate, but not NADH –supported respiration. (10; 16; 24; 30) In our study it is unlikely that NS1619 had a similar indirect role to inhibit complex II as a source for ROS generation because this effect was sensitive to paxilline (figure 5 B) and respiration was not slower with succinate + rotenone. Pyruvate has also been reported to decrease formation of H_2O_2 by changing the redox state (1) and to scavenge $OH^{\bullet}$. (9) In trial runs we observed that 30 μM NS1619 did enhance ROS release with α-ketoglutarate and glutamate as a NADH linked substrate. In our isolated heart model the presence of pyruvate in the perfusate did not effectively scavenge the upstream reactant, $O_2^{\bullet-}$, generated during ischemia and reperfusion. (19; 37) Another possibility is that the increase in ROS generated is elicited only by $FADH_2$ linked substrates. Reports by several laboratories indicate that complex II is a site of $O_2^{\bullet-}$ generation in mitochondria in addition to complex I and III. (23; 26; 44) It will be important to answer these questions in future studies: does mK_{Ca} channel activation induce ROS generation with NADH linked substrates, where are the sites of ROS generation induced by matrix K^+ influx and concomitant K^+/H^+ exchange, and which reactant ($O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, others) leads to preconditioning?

Possible limitations

Mitochondria isolated from their cells are not in a physiological environment as there is cross-talk between these compartments and ATP is consumed by cellular energy requirements. Respiration was decreased during state 3 by NS1619 and this was not blocked by paxilline, suggesting this is an effect independent on matrix K^+ influx or that these channels cannot be opened during state 3 respiration. This may indicate that these channels are normally open only during initial cell stress when mitochondria are in a reduced state. Another concern is that only under succinate + rotenone conditions was ROS generated. This may relate to the smaller baseline release of ROS with pyruvate than succinate as the substrate. As with the putative mK_{ATP} channel openers, there is a concern that the prominent protective effect of mBK_{Ca} channels openers is not actually mediated by increasing matrix K^+ influx, but by another mechanism. There is limited evidence for these channels in cardiac mitochondrial inner membrane. However, we too have found good coverage of protein fragments of BK_{Ca} alpha and beta subunits in several trypsinated purified preparations of guinea pig heart IMM using mass spectrometry (NP LC/ESI/MS) to the second profile (DF Stowe, unreported experiments). Finally, exogenous opening of this channel may give erroneous results compared to blocking endogenously opened channels because the agonist may have biphasic or unrelated effects at higher concentrations.

Summary and conclusions

Our results support the hypothesis that matrix K^+ influx through activated mBK_{Ca} channels modulates specific changes in mitochondrial bioenergetics, i.e., an imbalance of ψ_m to electron flow. Opening of mBK_{Ca} channels by NS1619 accelerates mitochondrial respiration during the resting states, but depolarizes ψ_m only at higher concentrations. Moreover, these conditions of accelerated respiration coupled with maintained ψ_m are suitable for allowing singlet electron leak capable of enhancing $O_2^{\bullet-}$ production. Thus, it is possible that the increase in respiration compensates for a reduced $\Delta\mu H$ due to mBK_{Ca} channel activation and K^+/H^+ exchange which maintains ψ_m. This mitochondrial mechanism may underlie, at least in part, the preconditioning effect of mK_{Ca} channel opening as a consequence of enhanced ROS generation.

ACKNOWLEDGEMENTS

The authors wish to thank the following for their assistance with these studies: Drs. Srinivasan G. Varadarajan, Ming Tao Jiang, Dan Beard, Janice Burke, and Meilin Huang, and also Michelle M. Henry, Anna Fekete, Anita Tredeau, James S. Heisner, and Richard Carlson, Jr.

REFERENCES

1. **Bassenge E, Sommer O, Schwemmer M and Bunger R**. Antioxidant pyruvate inhibits cardiac formation of reactive oxygen species through changes in redox state. *Am J Physiol Heart Circ Physiol* 279: H2431-H2438, 2000.
2. **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
3. **Brookes PS**. Mitochondrial H^+ leak and ROS generation: an odd couple. *Free Radic Biol Med* 38: 12-23, 2005.
4. **Brookes PS, Rolfe DF and Brand MD**. The proton permeability of liposomes made from mitochondrial inner membrane phospholipids: comparison with isolated mitochondria. *J Membr Biol* 155: 167-174, 1997.
5. **Cao CM, Xia Q, Gao Q, Chen M and Wong TM**. Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning. *J Pharmacol Exp Ther* 312: 644-650, 2005.
6. **Costa AD, Quinlan CL, Andrukhiv A, West IC, Jaburek M and Garlid KD**. The direct physiological effects of mitoK$_{ATP}$ opening on heart mitochondria. *Am J Physiol Heart Circ Physiol* 290: H406-H415, 2006.
7. **Debska G, Kicinska A, Dobrucki J, Dworakowska B, Nurowska E, Skalska J, Dolowy K and Szewczyk A**. Large-conductance K^+ channel openers NS1619 and NS004 as inhibitors of mitochondrial function in glioma cells. *Biochem Pharmacol* 65: 1827-1834, 2003.
8. **Debska G, Kicinska A, Skalska J, Szewczyk A, May R, Elger CE and Kunz WS**. Opening of potassium channels modulates mitochondrial function in rat skeletal muscle. *Biochim Biophys Acta* 1556: 97-105, 2002.
9. **Dobsak P, Courderot-Masuyer C, Zeller M, Vergely C, Laubriet A, Assem M, Eicher JC, Teyssier JR, Wolf JE and Rochette L**. Antioxidative properties of pyruvate and protection of the ischemic rat heart during cardioplegia. *J Cardiovasc Pharmacol* 34: 651-659, 1999.
10. **Dzeja PP, Bast P, Ozcan C, Valverde A, Holmuhamedov EL, Van Wylen DG and Terzic A**. Targeting nucleotide-requiring enzymes: implications for diazoxide-induced cardioprotection. *Am J Physiol Heart Circ Physiol* 284: H1048-H1056, 2003.
11. **Ferranti R, da Silva MM and Kowaltowski AJ**. Mitochondrial ATP-sensitive K^+ channel opening decreases reactive oxygen species generation. *FEBS Lett* 536: 51-55, 2003.
12. **Forbes RA, Steenbergen C and Murphy E**. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circ Res* 88: 802-809, 2001.
13. **Garlid KD**. Opening mitochondrial K$_{ATP}$ in the heart - what happens, and what does not happen. *Basic Res Cardiol* 95: 275-279, 2000.
14. **Garlid KD, Dos SP, Xie ZJ, Costa AD and Paucek P**. Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K^+ channel in cardiac function and cardioprotection. *Biochim Biophys Acta* 1606: 1-21, 2003.

15. **Garlid KD and Paucek P**. Mitochondrial potassium transport: the K^+ cycle. *Biochim Biophys Acta* 1606: 23-41, 2003.
16. **Hanley PJ and Daut J**. K_{ATP} channels and preconditioning: a re-examination of the role of mitochondrial K_{ATP} channels and an overview of alternative mechanisms. *J Mol Cell Cardiol* 39: 17-50, 2005.
17. **Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A and Terzic A**. Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. *Am J Physiol* 275: H1567-H1576, 1998.
18. **Holmuhamedov EL, Wang L and Terzic A**. ATP-sensitive K^+ channel openers prevent Ca^{2+} overload in rat cardiac mitochondria. *J Physiol* 519 Pt 2: 347-360, 1999.
19. **Kevin LG, Camara AK, Riess ML, Novalija E and Stowe DF**. Ischemic preconditioning alters real-time measure of O_2 radicals in intact hearts with ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 284: H566-H574, 2003.
20. **Korshunov SS, Korkina OV, Ruuge EK, Skulachev VP and Starkov AA**. Fatty acids as natural uncouplers preventing generation of $O_2^{\cdot-}$ and H_2O_2 by mitochondria in the resting state. *FEBS Lett* 435: 215-218, 1998.
21. **Korshunov SS, Skulachev VP and Starkov AA**. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 416: 15-18, 1997.
22. **Kowaltowski AJ, Seetharaman S, Paucek P and Garlid KD**. Bioenergetic consequences of opening the ATP-sensitive K^+ channel of heart mitochondria. *Am J Physiol Heart Circ Physiol* 280: H649-H657, 2001.
23. **Lenaz G**. The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. *IUBMB Life* 52: 159-164, 2001.
24. **Lim KH, Javadov SA, Das M, Clarke SJ, Suleiman MS and Halestrap AP**. The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration. *J Physiol* 545: 961-974, 2002.
25. **Liu Y, Sato T, O'Rourke B and Marban E**. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* 97: 2463-2469, 1998.
26. **McLennan HR and Degli Esposti M**. The contribution of mitochondrial respiratory complexes to the production of reactive oxygen species. *J Bioenerg Biomembr* 32: 153-162, 2000.
27. **Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DM and Sack MN**. Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res* 89: 787-792, 2001.
28. **Murata M, Akao M, O'Rourke B and Marban E**. Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca^{2+} overload during simulated ischemia and reperfusion: possible mechanism of cardioprotection. *Circ Res* 89: 891-898, 2001.
29. **O'Rourke B**. Evidence for mitochondrial K^+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.
30. **Ovide-Bordeaux S, Ventura-Clapier R and Veksler V**. Do modulators of the mitochondrial K_{ATP} channel change the function of mitochondria in situ? *J Biol Chem* 275: 37291-37295, 2000.
31. **Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV and Downey JM**. Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. *Circ Res* 87: 460-466, 2000.
32. **Riess ML, Camara AKS, Heinen A, Eells JT, Henry MM and Stowe DF**. Adenosine triphosphate sensitive potassium channel openers have opposite effects on mitochondrial respiration under different physiological conditions. *Biophys J* 88: 176a, 2005.

33. **Riess ML, Eells JT, Kevin LG, Camara AK, Henry MM and Stowe DF**. Attenuation of mitochondrial respiration by sevoflurane in isolated cardiac mitochondria is mediated in part by reactive oxygen species. *Anesthesiology* 100: 498-505, 2004.
34. **Sato T, Saito T, Saegusa N and Nakaya H**. Mitochondrial Ca^{2+}-activated K^+ channels in cardiac myocytes: a mechanism of the cardioprotective effect and modulation by protein kinase A. *Circulation* 111: 198-203, 2005.
35. **Shintani Y, Node K, Asanuma H, Sanada S, Takashima S, Asano Y, Liao Y, Fujita M, Hirata A, Shinozaki Y, Fukushima T, Nagamachi Y, Okuda H, Kim J, Tomoike H, Hori M and Kitakaze M**. Opening of Ca^{2+}-activated K^+ channels is involved in ischemic preconditioning in canine hearts. *J Mol Cell Cardiol* 37: 1213-1218, 2004.
36. **Starkov AA and Fiskum G**. Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. *J Neurochem* 86: 1101-1107, 2003.
37. **Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG and Jiang MT**. Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 290: H434-H440, 2006.
38. **Stowe DF and Riess ML**. Reactive oxygen species and cardiac preconditioning: many questions remain. *Cardiovasc Drugs Ther* 18: 87-90, 2004.
39. **Turrens JF, Alexandre A and Lehninger AL**. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 237: 408-414, 1985.
40. **Vanden Hoek TL, Becker LB, Shao Z, Li C and Schumacker PT**. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 273: 18092-18098, 1998.
41. **Wang X, Yin C, Xi L and Kukreja RC**. Opening of Ca^{2+}-activated K^+ channels triggers early and delayed preconditioning against I/R injury independent of NOS in mice. *Am J Physiol Heart Circ Physiol* 287: H2070-H2077, 2004.
42. **Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A and O'Rourke B**. Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* 298: 1029-1033, 2002.
43. **Yellon DM and Downey JM**. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 83: 1113-1151, 2003.
44. **Zhang L, Yu L and Yu CA**. Generation of superoxide anion by succinate-cytochrome c reductase from bovine heart mitochondria. *J Biol Chem* 273: 33972-33976, 1998.

Chapter 3

Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca^{2+} sensitive K^{+} channels

André Heinen, Mohammed Aldakkak, David F. Stowe, Samhita S. Rhodes, Matthias L. Riess, Srinivasan G. Varadarajan, and Amadou K.S. Camara

American Journal of Physiology - Heart and Circulatory Physiology
2007 Sep;293(3):H1400-7

ABSTRACT

Mitochondria generate reactive oxygen species (ROS) dependent on substrate conditions, O_2 concentration, redox state, and activity of the mitochondrial complexes. It is well known that the $FADH_2$-linked substrate succinate induces reverse electron flow to complex I of the electron transport chain and that this process generates superoxide ($O_2^{\cdot-}$); these effects are blocked by the complex I blocker rotenone. We demonstrated recently that succinate + rotenone-dependent H_2O_2 production in isolated mitochondria increased mildly on activation of the putative big mitochondrial Ca^{2+}-sensitive K^+ channel (mtBK$_{Ca}$) by low concentrations of 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS-1619). In the present study we examined effects of NS-1619 on mitochondrial O_2 consumption, membrane potential ($\Delta\psi_m$), H_2O_2 release rates, and redox state in isolated guinea pig heart mitochondria respiring on succinate but without rotenone. NS-1619 (30 μM) increased state 2 and state 4 respiration by 26 ± 4% and 14 ± 4%, respectively; this increase was abolished by the BK$_{Ca}$ channel blocker paxilline (5 μM). Paxilline alone had no effect on respiration. NS-1619 did not alter ($\Delta\psi_m$) or redox state but decreased H_2O_2 production by 73% vs. control; this effect was incompletely inhibited by paxilline. We conclude that under substrate conditions that allow reverse electron flow, matrix K^+ influx through mtBK$_{Ca}$ channels reduces mitochondrial H_2O_2 production by accelerating forward electron flow. Our prior study showed that NS-1619 induced an increase in H_2O_2 production with blocked reverse electron flow. The present results suggest that NS-1619-induced matrix K^+ influx increases forward electron flow despite the high reverse electron flow, and emphasize the importance of substrate conditions on interpretation of effects on mitochondrial bioenergetics.

INTRODUCTION

Mitochondria are known to generate reactive oxygen species (ROS), which include superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) as byproducts of aerobic metabolism (3; 34). Excess release of ROS has been shown to play a role in the etiology of various pathological disorders including cardiovascular disease (1), degenerative changes in aging (5), Alzheimer's disease (28), and diabetes (22), as well as in ischemia/reperfusion (IR) injury (41). ROS are key elements in a variety of cellular signaling pathways (24), including cardioprotection against IR induced by ischemic and pharmacological preconditioning (26; 30).

$O_2^{\cdot-}$ can be generated at several sites along the mitochondrial electron transport chain (ETC) including complex III (12; 35) and complex I (8; 18; 20). Complex III generates $O_2^{\cdot-}$ through the oxidation of ubisemiquinone, a radical intermediate formed through the cycle in the complex. The Q_o site of the cycle is a major source of $O_2^{\cdot-}$ production and it is close to the intermembrane space. In contrast, $O_2^{\cdot-}$ generated from complex I is released into the matrix. On study (20) suggests that the primary site of $O_2^{\cdot-}$ generation in the mitochondrial ETC is flavin mononucleotide (FMN) of complex I via reverse electron flow, not forward electron flow via ubiquinone of complex III. Whatever the source of $O_2^{\cdot-}$ is, the mechanism and quantity of $O_2^{\cdot-}$ generated is dependent on the experimental substrate and energetic conditions (18; 21; 36). When $FADH_2$ related substrates are used and electrons enter the ETC at complex II (succinate dehydrogenase), $O_2^{\cdot-}$ can be generated by reverse electron flow to complex I (21). The resulting large increase in $O_2^{\cdot-}$ generation is dependent on a high inner mitochondrial membrane (IMM) potential ($\Delta\psi_m$), and is sensitive to complex I blockade by rotenone, which prevents reverse electron flow as a source of $O_2^{\cdot-}$ generation (18; 21). It is believed that in the presence of a high proton motive force electrons are passed to NAD^+ until the pool is fully reduced to NADH; once this occurs semiquinone can only lose its unpaired electron to O_2 because all upstream redox centers are fully reduced (18).

K^+ channels located in the IMM appear to play an important role in regulating mitochondrial function (15; 24), but the mechanism remains unclear. Xu et al. (40) found evidence for big Ca^{2+}-sensitive K^+ ($mtBK_{Ca}$) channels in the IMM of guinea pig ventricular cells. Sato et al. (31) demonstrated that opening of $mtBK_{Ca}$ channels increases flavoprotein oxidation in ventricular myocytes placed in glucose-free Tyrode's solution, indicating an increase in electron transport in oxidized mitochondria. Recently, we investigated the effects of $mtBK_{Ca}$ channel opening and closing on function of mitochondria isolated from

guinea pig hearts (16). We reported that putative mtBK$_{Ca}$ channel opening with low concentrations of NS-1619 accelerated states 2 and 4 respiration (electron flow) and H_2O_2 generation at a stable $\Delta\psi_m$ in the presence of succinate and rotenone (16). In the present study we investigated effects of NS-1619 on respiration, $\Delta\psi_m$, redox state (NADH and FAD), and H_2O_2 generation using succinate alone, which can induce ROS generation via reverse electron flow. We proposed that under these conditions H_2O_2 production would decrease because of a relative increase in forward electron flow, induced by matrix K^+ influx, thus countering the larger reverse electron flow caused by succinate with subsequent $O_2^{\cdot-}$ formation at complex I.

MATERIALS AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health No. 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committee (Medical College of Wisconsin, Milwaukee, Wisconsin).

Mitochondrial isolation

Heart mitochondria were isolated from ketamine-anesthetized guinea pigs (250-300 g) by differential centrifugation as described previously (29) with moderate modifications. Briefly, ventricles were excised, placed in an isolation buffer: 200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 5 mM 3-(n-morpholino) propranesulfonic acid (MOPS), 1 mM EGTA, 0.1% bovine serum albumin (BSA), pH 7.15 (adjusted with KOH), and minced into 1 mm^3 pieces. The suspension was initially homogenized for 15 s in 2.5 ml isolation buffer containing 5 U/ml protease, and for another 15 s after addition of 17 ml isolation buffer. The suspension was centrifuged at 8000*g* for 10 min; the pellet was resuspended in 25 ml isolation buffer and centrifuged again at 750*g* for 10 min. Next, the supernatant was centrifuged at 8000*g* for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. The protein content was determined by the Bradford method (4). All isolation procedures were conducted at 4°C.

Mitochondrial O_2 consumption

Oxygen consumption was measured polarographically at 27^{o}C using a respirometry system (System S 200A; Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.25 mg protein/ml) were suspended in respiration buffer containing 130 mM KCl, 5 mM K_2HPO_4,

20 mM MOPS, 2.5 mM EGTA, 1 µM $Na_4P_2O_7$, 0.1% BSA, pH 7.15 adjusted with KOH. Buffer [Ca^{2+}] was less than 100 nM as assessed by the fluorescence dye indo-1. Respiration was initiated by administration of complex II substrate succinate (10 mM). State 3 respiration was determined after addition of 250 µM ADP, and state 4 respiration was measured after complete phosphorylation of ADP to ATP. The respiratory control index (RCI) was calculated as the ratio of mean slopes during state 3 and state 4 respiration (state 3 slope/state 4 slope).

Mitochondrial H_2O_2 release rate

Rates of mitochondrial H_2O_2 release were measured spectrophotometrically (QM-8, Photon Technology International, PTI) at 27°C following oxidation of amplex red (25 µM; Molecular Probes) to the highly fluorescent product resorufin in the presence of 0.1 U/ml horseradish peroxidase. Excitation and emission wavelengths (λ_{ex} and λ_{em}) were set to 530 nm and 583 nm, respectively. Mitochondria (0.5 mg/ml) were suspended in respiration buffer. Time-controls received 0.3% dimethyl sulfoxide (DMSO). Maximal H_2O_2 production was stimulated in some experiments by addition of complex III blocker antimycin A (5 µM). Antimycin A is believed to inhibit cytochrome *b* oxidation by cytochrome c_1 to cause accumulation of ubisemiquinone, which is oxidized by molecular O_2 to generate $O_2^{\bullet-}$ and H_2O_2 (7). An additional increase in ubisemiquinone may be attributed to $\Delta\psi_m$ depolarization by the protonophore CCCP, and the consequent increase in electron transfer reactions between complex II (succinate dehydrogenase) and ubiquinone and ubiquinol, and cytochrome c_1. Catalase (300 U/ml) was added to confirm H_2O_2 production by attenuating the H_2O_2 signal, since catalase converts H_2O_2 to H_2O. H_2O_2 release rates were expressed as arbitrary fluorescence units (afu, Figures 4 and 5A) or as percent of time control experiments (Figure 5B). Baseline H_2O_2 levels were calibrated from a mean of 3 standard curves of photon counts over a range of 10-200 nM H_2O_2 (added to assay medium in the presence of reactants amplex red and horseradish peroxidase); each regression was linear ($R > 0.99$).

Mitochondrial redox state

Mitochondrial redox state was measured by the autofluorescence that arises from compounds endogenous to the mitochondrion. The NAD^+ signal is not fluorescent, but an increase in NADH fluorescence signal reflects an increase in the ratio of NADH to NAD^+, i.e, a net shift in the pyridine nucleotide pool to the reduced state. In contrast to NADH, a decrease in the FAD signal (flavoprotein fluorescence) occurs when the carrier binds to

electrons. Thus a decrease in FAD reflects an increase in the ratio of reduced to oxidized flavoprotein (14). NADH and FAD were measured from the same aliquot of mitochondrial suspension (0.5 mg/ml) in respiratory buffer, with the aid of an electronic chopper that switched between the excitations for NADH and FAD so that the time resolution for the three NADH and FAD emission signals was 7 s. NADH was determined by exciting at 350 nm λ_{ex} and recording at 460 nm λ_{em} and 405 nm λ_{em} (the latter reference wavelength is less sensitive to NADH changes). The fluorescence ratio, F460/F405, is interpreted as a measure of NADH. Mitochondrial FAD autofluorescence was recorded at 540 nm λ_{em} from light filtered at 480 nm λ_{ex}.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) was monitored at 27°C in a cuvette- based spectrophotometer (QM-8, PTI) operating 503 nm λ_{ex} and 527 nm λ_{em}, respectively, in the presence of the fluorescence dye rhodamine 123 (50 nM). Mitochondria (0.5 mg/ml) were suspended in respiration buffer. $\Delta\psi_m$ was expressed as the percentage of rhodamine 123 fluorescence in the presence of fully coupled mitochondria relative to the fluorescence after addition of 4 μM of the uncoupler carbonyl-cyanide-m-chlorophenylhydrazenone (CCCP). To verify the functional integrity of mitochondria, 250 μM ADP was added and repolarization of $\Delta\psi_m$ after complete phosphorylation of ADP was measured.

Chemicals and reagents

Rhodamine 123, amplex red, and indo-1 were purchased from Molecular Probes (Eugene, OR) and high purity KCl from EMD Chemicals (Gibbstown, NJ). All other chemicals were purchased from Sigma Chemical Co. NS-1619, paxilline, and amplex red were dissolved in DMSO before adding to the experimental buffer.

Statistical analyses

Group data were compared by analysis of variance. If F values ($P < 0.05$) were significant, post hoc comparisons of means tests (Student-Newman-Keuls) were considered statistically significant when $P < 0.05$ (two-tailed); * vs. control; $^{\#}$ vs. NS-1619. Data are presented as means ±SEM.

RESULTS

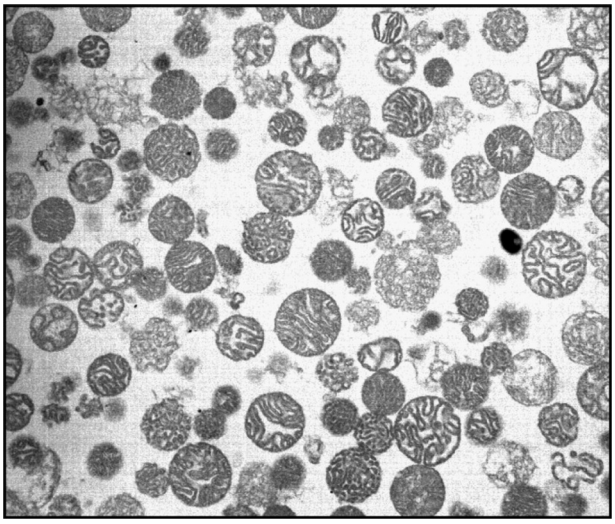
Mitochondrial integrity after isolation

The morphological integrity of mitochondria after the isolation procedure was verified by electron microscopy. Figure 1 shows guinea pig heart isolated mitochondria with intact inner and outer membranes suspended in isolation buffer. A RCI of 2.5±0.1 in the control group with succinate as substrate demonstrated strong coupling of respiration and oxidative phosphorylation. This indicates functioning mitochondria with integrity of the respiratory complexes in the IMM after the isolation procedure.

Mitochondrial respiration

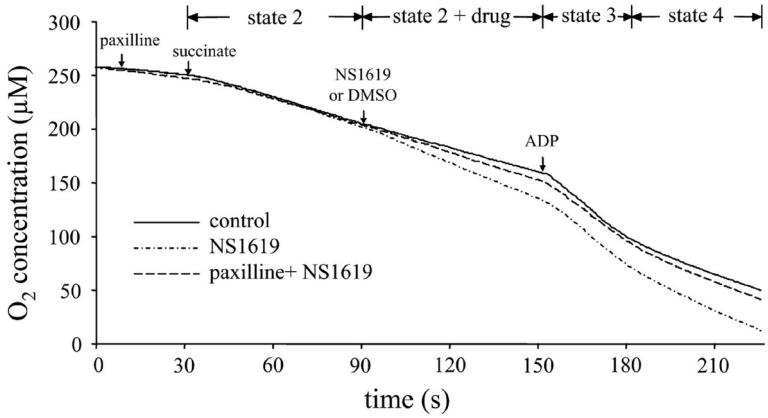
The experimental procedure and sample traces for respiration experiments are shown in figure 2. Opening of putative $mtBK_{Ca}$ channels by 30 μM NS-1619 significantly increased state 2 respiration by 26±4% over control and state 4 respiration by 14±4% over control (Figures 3A and 3C).

Figure 1:



Electron microscopy to confirm the high morphological integrity of heart mitochondria after the isolation procedure

Figure 2: Representative traces of mitochondrial respiration experiments



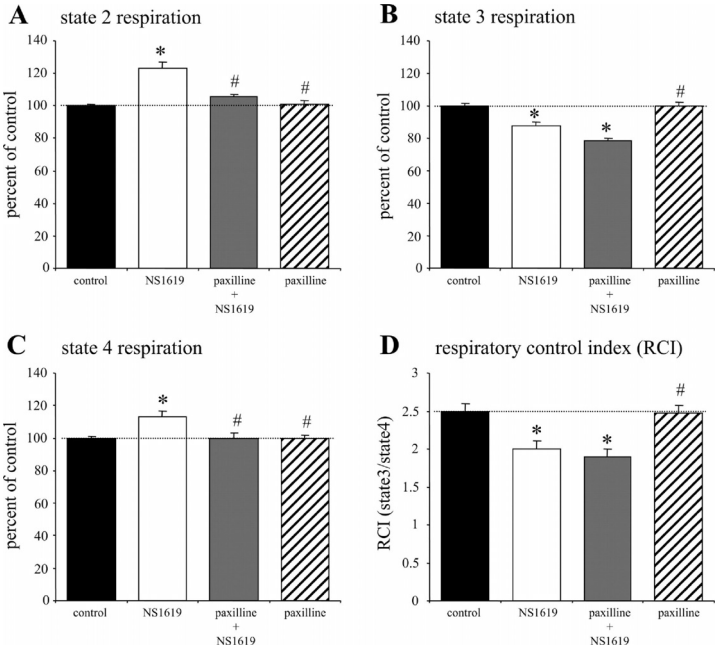
State 2 respiration was initiated by addition of 10 mM succinate; state 3 respiration was initiated by addition of 250 µM ADP. NS-1619 or its vehicle DMSO (0.3%) was administered at ~90 s (treatment effects on state 2 were measured beginning at 120 s) in the presence or absence of 5 µM paxilline.

To verify that the increase in state 2 and state 4 respiration was due to mtBK$_{Ca}$ channel opening by NS-1619, 5 µM paxilline was added in the absence or presence of 30 µM NS-1619 (Figure 3). Pre-administration of paxilline significantly blocked the NS-1619 -induced increase in state 2 (to 4±3% from 26±4%) and state 4 respiration (to -1±3% from 14±4%). Paxilline alone had no significant effect on respiration (0±2%), which indicates that mtBK$_{Ca}$ channels were closed under these experimental conditions. State 3 respiration was decreased statistically by NS-1619 (-12±3% vs. control), but co-administration of paxilline reduced but did not significantly block this effect (-21±2%) (Figure 3B), which may indicate a mtBK$_{Ca}$ channel independent effect. These data demonstrate that a low concentration of NS-1619 increases succinate -supported respiration, but only during the resting states when the basal respiratory rate is low.

Reversed electron flow induced H_2O_2 generation

To verify that the major mechanism of ROS production with the complex II substrate succinate is due to reverse electron flow, we measured mitochondrial H_2O_2 release rate.

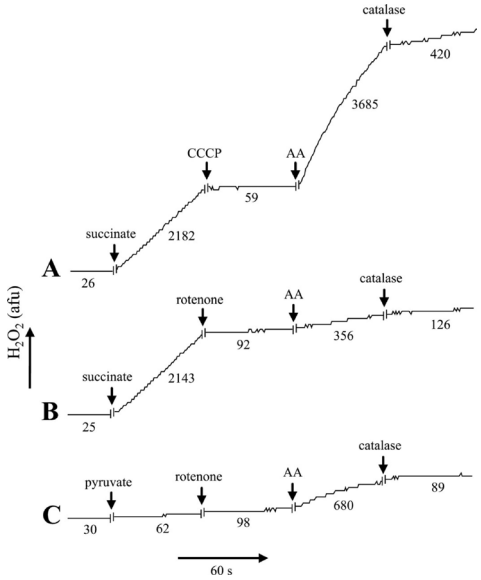
Figure 3:



Summarized data for the effects of 30 μM NS-1619 and the antagonist effects on big mitochondrial Ca^{2+}-sensitive K+ channel ($mtBK_{Ca}$) opening by 5 μM paxilline on mitochondrial respiration in the presence of the complex II substrate succinate (10 mM). *P < 0.05 vs. control; #P < 0.05 vs. NS-1619 (n = 10 for each group).

In representative tracings figure 4 shows that succinate initiated a large increase in the H_2O_2 release rate that was abolished by either the mitochondrial uncoupler CCCP (panel A) or the complex I blocker rotenone (panel B). This verified in our model that reversed electron flow into complex I of the ETC is the main mechanism by which $O_2^{\cdot-}$ is generated with succinate alone as the substrate. Moreover, the rate of H_2O_2 release in succinate -supported respiration was approximately 35 times higher than the rate attained by the complex I substrate pyruvate (panel C). Antimycin A, a complex III blocker, increased the H_2O_2 release rate much more after uncoupling with CCCP than after blocking complex I with rotenone.

Figure 4: Mitochondrial H_2O_2 release rate



Representative traces of H_2O_2 release rates during succinate (10 mM)-supported respiration. H_2O_2 generation was abrogated by addition of 4 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; *A*), a mitochondrial uncoupler, or 4 µM rotenone (*B*), a complex I blocker. *C*: H_2O_2 generation rate during pyruvate (complex I substrate, 10 mM)-supported mitochondrial respiration. Catalase (300 U/ml) was added to confirm H_2O_2 production. AA, antimycin A (complex III blocker; 5 µM); afu, arbitrary fluorescence units. Numbers represent changes in afu per minute.

Effect of mtBK$_{Ca}$ channel opening on H_2O_2 generation

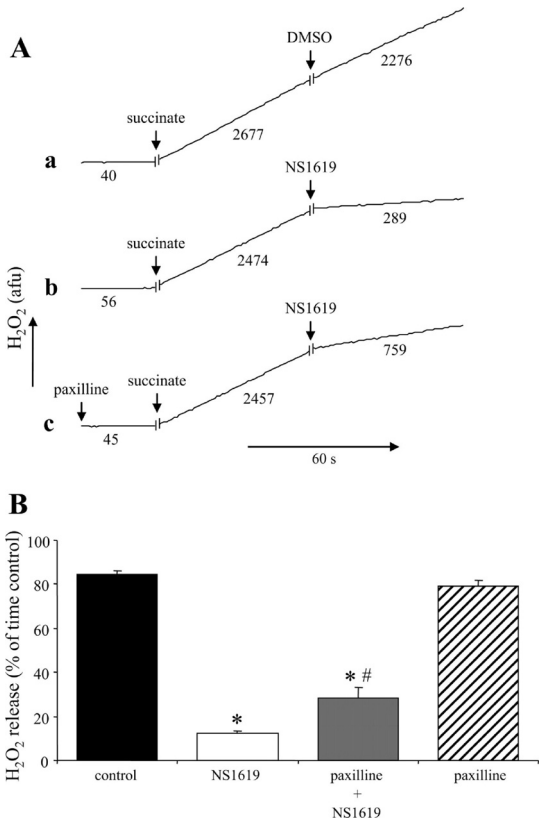
To investigate the effect of mtBK$_{Ca}$ channel opening on reverse electron flow -induced H_2O_2 production we measured the H_2O_2 release rate after addition of NS-1619 in succinate -supported mitochondria. Results are expressed as percent of time controls. Figure 5 shows that addition of NS-1619 significantly decreased the H_2O_2 release rate from 85±2% to 12±1%. Pre-administration of paxilline statistically attenuated this reduction in ROS generation (28±5% vs. 12±1%). Paxilline alone had no significant effect on mitochondrial H_2O_2 release rate (79±2% vs 85±2%).

Effect of NS-1619 on mitochondrial redox state

In this *in vitro* preparation, oxidation of succinate and reduction of molecular O_2 involves in part, reverse electron flow, specifically of electrons entering at complex II to react with

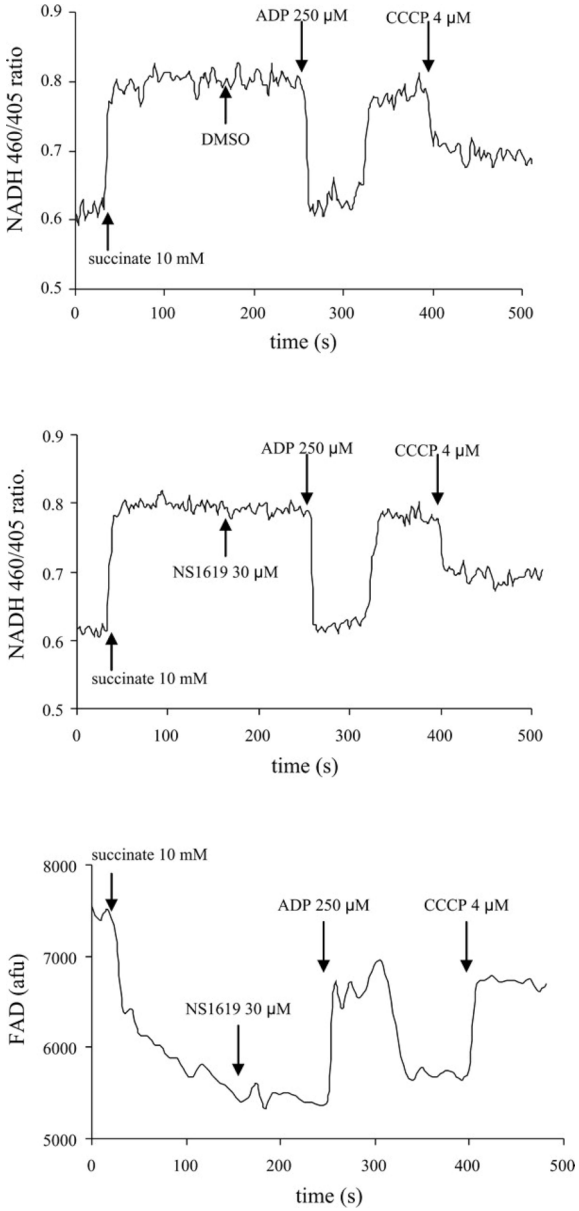
NAD^+ to produce NADH at complex I. As shown in Figure 6, addition of succinate invariably increased NADH and decreased FAD. NADH also increases while FAD decreases with pyruvate, a complex I substrate, but this occurs because pyruvate directly reduces NAD^+ to NADH with forward electron flow. To confirm that NADH and FAD signals reflect changes in redox state, we noted that ADP transiently decreased NADH and increased FAD (state 3) whereas CCCP, a protonophore uncoupler, maximally oxidized mitochondria as observed by the low NADH and high FAD states.

Figure 5: Mitochondrial H_2O_2 release rate



A: representative traces for H_2O_2 release rate with succinate as substrate: control (*a*), 30 μM NS-1619 (*b*), 5 μM paxilline + 30 μM NS-1619 (*c*). *B*: summarized data for H_2O_2 release rate as % of time controls. A 10% change represents a change in H_2O_2 release rate of ~1.5 pmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein. All treatment effects are compared with the baseline of the same experiment. *P < 0.05 vs. control; #P < 0.05 vs. NS-1619; n = 6 for each group

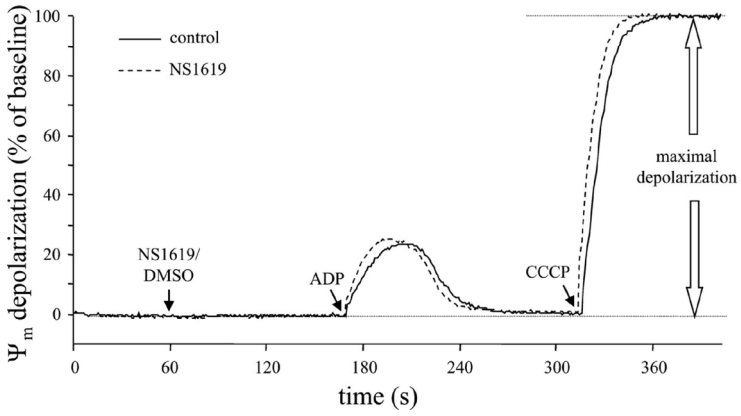
Figure 6: Effects of NS-1619 on mitochondrial redox state (NADH and FAD)



Representative traces for FAD (*bottom*) and NADH (460/405-nm emission wavelength; *top*). Concentrations up to 30 µM NS-1619 had no effect to decrease the redox state as did ADP and CCCP; higher concentrations decreased the NADH but not the FAD redox state (see text).

Importantly, NS-1619, 30 μM, (and lower concentrations, not shown) did not significantly alter either NADH (1.2 ±1.3%) or FAD (0.8 ±1.4%) compared to the vehicle (0%) (P>0.05, n=6); however, 50 and 100 μM NS-1619 significantly and dose-dependently decreased NADH to 18± 2%, and 98 ±3% (P<0.05, n=3) of the response to ADP (100%); these higher concentrations, however, did not alter FAD (-2 ±3% vs control; P<0.05, n=3), reflecting its independence of the NADH redox state.

Figure 7:



Effects of NS-1619 on inner mitochondrial membrane potential ($\Delta\psi_m$): representative traces for $\Delta\psi_m$ with succinate as substrate. NS-1619 (30 μM) or its vehicle DMSO (0.3%) was added. To verify a highly charged $\Delta\psi_m$ and the functional integrity of mitochondria, 250 μM ADP was added as indicated. Maximal depolarization was measured after addition of 4 μM CCCP to uncouple oxidative phosphorylation.

Mitochondrial membrane potential ($\Delta\psi_m$)

To examine if the effects of $mtBK_{Ca}$ channel opening on reducing ROS generation were due to depolarization of the IMM, we measured fluorescence of the $\Delta\psi_m$ -sensitive dye rhodamine 123 (Figure 7).

Administration of 30 μM NS-1619 had no significant effect on $\Delta\psi_m$ during succinate supported respiration (0.2±0.1% vs. 0.3±0.1%, P>0.05, n=6; summary data not displayed).

DISCUSSION

The major conclusions of this study are that during succinate-supported respiration *1*) $mtBK_{Ca}$ channel activation by 30 μM NS-1619 decreases reverse electron flow-induced

H_2O_2 production, which is attenuated by paxilline, and *2*) this reduction in H_2O_2 production by NS-1619 is not due to depolarization of $\Delta\psi_m$ or to a more oxidized redox state. The relative decrease in reverse electron flow, and thereby $O_2^{\cdot-}$ generation, is likely due to NS-1619-enhanced forward electron flow.

Reverse electron flow as a mechanism for $O_2^{\cdot-}$ generation

The ETC is the main source of $O_2^{\cdot-}$ generation during normal metabolism (19). Any reduced metal ion component of the ETC can serve as a potential source of $O_2^{\cdot-}$ by one-electron transfer to O_2 to generate $O_2^{\cdot-}$ (10). With the use of specific respiratory complex blockers, $O_2^{\cdot-}$ has been shown to be released into the matrix or cytosol at complex III and into the matrix at complex I (12). In turn, $O_2^{\cdot-}$ is dismutated by superoxide dismutase in the matrix (SOD2) or cytosol (SOD1) to form H_2O_2, most of which is detoxified to H_2O by the glutathione system. However, H_2O_2 is also a progenitor of the highly reactive $^{\cdot}OH$ in the presence of a reduced transition metal such as Fe^{2+}. Since H_2O_2 is highly permeable through the IMM, the H_2O_2 measured in this study likely reflects its generation in the matrix.

Several studies have reported that $O_2^{\cdot-}$ generation is greater during respiration supported with $FADH_2$-linked substrates than with complex I substrates (21; 36). $O_2^{\cdot-}$ generation with the complex II substrate succinate is caused by reverse electron flow into complex I of the ETC and is largely dependent on a high $\Delta\psi_m$ (20; 21). This is supported by our observation that uncoupling of mitochondria with CCCP, which collapses $\Delta\psi_m$ by allowing proton reentry through the IMM, halted H_2O_2 generation. Moreover, the complex I blocker rotenone and ADP (state 2-to-state 3 transition) largely attenuated reverse electron flow-induced H_2O_2 generation, as also shown by others (20; 21; 36). In hearts utilizing NADH-linked substrates, blocking electron flow at complex I reduces mitochondrial damage during I/R injury at least in part because of reduced ROS production (11).

Possible significance of reverse electron flux

Significant $O_2^{\cdot-}$ generation occurs via forward electron flow in the presence of complex I Q site inhibitors like rotenone, but much more is generated during reverse electron flow through complex I. Lambert and Brand (18) argue that the site of generation by complex I is likely a ubisemiquinone-binding site rather than upstream flavin or FeS centers. Reverse electron flux may be a significant factor in I/R injury. Physiologically, succinate is synthesized at low concentrations (0.2–0.4 mM) inside mitochondria in vivo and is not a natural substrate. However, it rises substantially during ischemia or hypoxia (up to 4–7 mM) (20). It is possible that during early ischemia, when NADH levels are high, and during

initial reperfusion, the oxidation of accumulated succinate generates the high $\Delta\psi_m$ and reduces power necessary for reversal of electron transfer and $O_2^{\cdot-}$ generation at complex I. In phosphorylating mitochondria respiration is controlled by both ATP turnover and electron supply. Adenine nucleotide translocase (ANT) is an important site of control in oxidative phosphorylation (14) because it catalyzes the one-for-one exchange of adenine nucleotides. In energized mitochondria, ANT preferentially ejects more ATP than ADP brought into the matrix. This would lead to a greater extramitochondrial ATP-to-ADP ratio, which could lead to activation of succinate dehydrogenase (complex II) and stimulation of reverse electron flow.

Effects of NS-1619 and paxilline on BK_{Ca} channel

NS-1619, a benzimidazole derivative, promotes opening of high-conductance (300 pS) BK_{Ca} channels in membranes of a wide variety of cell types (33). NS-1619-induced effects on smooth muscle can be blocked by charybdotoxin or paxilline, but not by glibenclamide, which indicates that the action of NS-1619 in plasma membranes is predominantly on the BK_{Ca} channel. The rapid effect of NS-1619 suggests that its mechanism of action is either directly on the channel protein itself or on a closely associated modulatory protein (27). The absence of intracellular Ca^{2+} prevents BK_{Ca} channel activation by NS-1619, and the drug may increase channel activation by making the channel more sensitive to intracellular $[Ca^{2+}]$ (27). The BK_{Ca} channels are tetramers of a pore-forming α-subunit of the *slo* gene family and a regulatory β -subunit, which is structurally unique and transmembrane spanning; the α-subunit, encoded by a single gene, is comprised of seven transmembrane segments and four intracellular hydrophobic domains (17; 23; 38). Several groups are attempting to more clearly identify and characterize these channels in cardiac IMM.

Modulation of mitochondrial function by $mtBK_{Ca}$ channels

Xu et al. (40) first reported evidence for $mtBK_{Ca}$ channels in the IMM of guinea pig ventricular cells. Patch-clamp recordings from mitoplasts of these cells showed Ca^{2+}-dependent, large-K^+ conductance channels in the IMM, and immunoblots of cardiac mitochondria with antibodies against the COOH-terminal part of BK_{Ca} channel identified a 55-kDa protein as part of this putative channel. The β_1-subunit of the $mtBK_{Ca}$ channel, as tentatively identified in the IMM, interacts with the cytochrome-*c* oxidase subunit I (25). The binding sites for charybdotoxin and NS-1619 are likely in the cytosolic compartment, whereas sites for Ca^{2+} are likely on the matrix side of the IMM (39).

mK_{Ca} channels and reversed electron flow induced ROS production

The ultimate bioenergetic modulating effects of K^+ influx into the mitochondrial matrix through K^+ channels, including both $mtBK_{Ca}$ and mitochondrial ATP-sensitive K^+ channels (mtK_{ATP}), however, remains unclear. Sato et al. (31) demonstrated that NS-1619 increases flavoprotein oxidation in ventricular myocytes placed in glucose-free Tyrode solution; this suggested an increase in electron transport in oxidized mitochondria. Recently, we reported (16) on the concentration-dependent effects of NS-1619 on respiration, $\Delta\psi_m$, and H_2O_2 generation in isolated guinea pig heart mitochondria respiring on the complex II substrate succinate in the presence of the complex I blocker rotenone to prevent reverse electron flow. NS-1619 increased state 2 and state 4 respiration, effects that were inhibited by paxilline. These findings are in agreement with the hypothesis of O'Rourke (24), who suggested that mitochondrial K^+ channels function as energy (stored as the proton gradient, $\Delta\mu_H$)-dissipating channels by expending $\Delta\mu_H$, in part, to eject K^+ that enters the matrix through activated K^+ channels via an electroneutral K^+/H^+ exchanger. This decrease in $\Delta\mu_H$ would stimulate respiration to compensate for net proton leak, with the consequence of a maintained $\Delta\psi_m$ (16). In the present report we demonstrated during succinate-supported respiration that putative $mtBK_{Ca}$ channel opening by 30 µM NS-1619, and by inference by lower concentrations, again increased state 2 and state 4 respiration and had no effect on redox state or $\Delta\psi_m$.

Cancherini et al. (9) reported recently that NS-1619 stimulated nonphosphorylating respiration (state 4) and inhibited ADP-stimulated respiration (state 3) in isolated rat heart mitochondria. These effects of NS-1619 were also described previously by Debska et al. (13) and by our group (16) and are confirmed again in this study in guinea pig heart mitochondria. However, in the former study (9) evidence was presented that NS-1619 does not specifically transport K^+ via a channel or cation transporter. In the presence of the complex V inhibitor oligomycin they reported that NS-1619 depolarized $\Delta\psi_m$ in K^+-containing as well as K^+-free buffer, that the respiratory effects were not blocked by paxilline, that NS-1619-induced matrix swelling occurred also in a tetraethylammonium-based buffer, and that the latter effect was not blocked by paxilline. On the basis of these findings Cancherini et al. (9) suggested that NS-1619 promotes nonselective permeabilization of the IMM to ions rather than acting on a specific IMM K^+ channel. However, in this and our prior study (16), the effect of NS-1619 to enhance state 4 respiration was inhibited by paxilline, a known BK_{Ca} channel inhibitor, and uncoupling did not occur at less than 30 µM NS-1619.

From these pharmacological results, we conclude that the NS-1619-induced increase in state 4 respiration, and the state- and substrate-dependent effects on H_2O_2 production, are

likely $mtBK_{Ca}$ channel mediated. We cannot reconcile differences between these studies, but we agree that the specificity of NS-1619 for the putative $mtBK_{Ca}$ channel remains speculative. Moreover, the putative $mtBK_{Ca}$ channel will need to be better identified and characterized in the IMM to substantiate its role in modulating mitochondrial bioenergetics. What is evident to us, however, is that NS-1619 clearly initiates pharmacological preconditioning against cardiac I/R injury and that this protective effect is effectively blocked by a $O_2^{\cdot-}$ dismutase mimetic as well as by paxilline (32).

Effect of putative $mtBK_{Ca}$ channel opening on reverse electron flow-induced H_2O_2 production

Under physiological conditions reverse electron flow does not occur, because forward electron flow through complex I via NADH prevents it. However, under pathophysiological conditions in which NADH is depleted, reverse electron flow may lead to $O_2^{\cdot-}$ generation at complex I (6; 34; 36). In succinate-supported isolated mitochondria $O_2^{\cdot-}$ generation due to reverse electron flow to complex I is dependent on a fully charged $\Delta\psi_m$ under state 4 conditions; reverse flow is blocked by the complex I blocker rotenone. Our results suggest that reverse electron flow-induced H_2O_2 production can be modulated by matrix K^+ flux. The H_2O_2 release rate during enhanced state 4 respiration by NS-1619 can either increase (no reverse electron flow) (16), or decrease (reverse electron flow), as shown in this report. The changes in H_2O_2 production were not caused by an effect of NS-1619 on $\Delta\psi_m$ or redox state, which did not change. Thus we propose that matrix K^+ influx through activated $mtBK_{Ca}$ channels can reduce H_2O_2 production due to reverse electron flow by accelerating forward electron flow. This is in agreement with a previous finding by Votyakova and Reynolds (37), who demonstrated that very low concentrations of the mitochondrial uncoupler FCCP, which did not depolarize $\Delta\psi_m$, were sufficient to reduce reverse electron flow-induced $O_2^{\cdot-}$ generation.

That NS-1619, at concentrations at or below 30 μM, did not alter the mitochondrial redox state is consistent with our previous finding with pyruvate or succinate + rotenone (16), in which $\Delta\psi_m$ remained high, a condition that can lead to enhanced $O_2^{\cdot-}$ generation and downstream reactants. Reverse electron flow-induced $O_2^{\cdot-}$ generation depends on the delivery of electrons downstream of complex I, i.e., from complex II, a high proton motive force, and a low redox potential across complex I (i.e., no pyruvate to increase the NADH-to-NAD^+ ratio). Thus a factor that limits reverse electron flow-induced H_2O_2 production is supply of NADH by pyruvate at complex I, which in its absence is supported by NADH generation from NAD^+ by succinate at complex I. Indeed, Liu et al. (20) showed that

inhibition of complex II with malonate completely abolished the succinate-induced reduction of NAD^+. This suggested that electrons for reducing NAD^+ come directly from succinate through reverse electron transport and not from other components of the tricarboxylic acid cycle (20). The specific site for $O_2^{\cdot-}$ generation under physiological or pathological conditions may be the FMN group of complex I (20) or a ubisemiquinone-binding site (18).

Batandier et al. (2) proposed that with succinate as substrate, any decrease in NADH level or $\Delta\psi_m$ would abolish $O_2^{\cdot-}$ generation at this site. Such a scenario is partially in agreement with our study, in which we show that NADH decreased, while FAD did not change, at higher, but not lower, NS-1619 concentrations. It is notable that rotenone blocks succinate-supported NADH formation via reverse electron flow, but also blocks oxidation of NADH by directly inhibiting complex I. The end result is that with rotenone NADH increases during oxidation of succinate because succinate is converted to malate, which generates NADH that feeds into complex I.

The question of how modulation of mitochondrial function by mtBK$_{Ca}$ channel opening might differentially alter $O_2^{\cdot-}$ generation depending on substrate conditions requires further study. Under the same O_2 tension conditions, mitochondria appear to generate $O_2^{\cdot-}$ by different mechanisms: *1*) by accelerating resting state respiration, e.g., due to mtBK$_{Ca}$ channel activation or valinomycin-induced matrix K^+ influx, under forward electron flow conditions while $\Delta\psi_m$ is maintained, and *2*) by reverse electron flow into complex I during succinate-supported respiration with no fall in $\Delta\psi_m$. However, we could not demonstrate NS-1619-induced H_2O_2 production with the substrate pyruvate (16). We propose that H_2O_2 production decreased with the increase in NS-1619-induced respiration in the presence of succinate because of a compensatory outward flux of K^+ and influx of protons via K^+/H^+ exchange, which accelerates forward electron flow, thus reducing the impact of reverse flow on $O_2^{\cdot-}$ generation. Clearly, the consequences of altered matrix K^+ flux likely alter the flux of other cations in addition to H^+, for example, Na^+ and Ca^{2+} by Na^+/H^+ and Na^+/Ca^{2+} exchange in the IMM.

In summary, we report that in fully membrane-polarized and reduced mitochondria matrix K^+ influx can either increase or decrease $O_2^{\cdot-}$ generation depending on substrate conditions. This work emphasizes the impact of experimental substrate conditions when analyzing mitochondrial bioenergetics, and may help to explain some of the conflicting results in the literature regarding the effect of K^+ channel activation and matrix K^+ flux on modulating mitochondrial function and H_2O_2 production. Moreover, caution must be taken on the effect of NS-1619 to open mtBK$_{Ca}$ channels or of paxilline to block them because although

paxilline completely reversed the increase in respiration, it only incompletely blocked the decrease in H_2O_2 production. We must have a precise identification and characterization of the several putative mitochondrial K^+ channels and a better grasp of the pharmacology of the drugs used to explore these mechanisms. Much research remains to be done to understand the physiological (cell conditioning) and pathological (cell damage) conditions by which matrix K^+ flux modulates matrix pH, respiration, and $O_2^{\cdot-}$ generation.

ACKNOWLEDGEMENTS

The authors thank the following for assistance with these studies: Anita Tredeau, Ming Tao Jiang, Michelle M. Henry, Anna Fekete, Janice Burke, James S. Heisner, Richard Carlson, Jr., Dan Beard, and Meilin Huang.

REFERENCES

1. **Ballinger SW**. Mitochondrial dysfunction in cardiovascular disease. *Free Radic Biol Med* 38: 1278-1295, 2005.
2. **Batandier C, Guigas B, Detaille D, El-Mir MY, Fontaine E, Rigoulet M and Leverve XM**. The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. *J Bioenerg Biomembr* 38: 33-42, 2006.
3. **Boveris A and Cadenas E**. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Lett* 54: 311-314, 1975.
4. **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
5. **Brand MD, Buckingham JA, Esteves TC, Green K, Lambert AJ, Miwa S, Murphy MP, Pakay JL, Talbot DA and Echtay KS**. Mitochondrial superoxide and aging: uncoupling-protein activity and superoxide production. *Biochem Soc Symp* 203-213, 2004.
6. **Brookes PS, Yoon Y, Robotham JL, Anders MW and Sheu SS**. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287: C817-C833, 2004.
7. **Cadenas E and Boveris A**. Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* 188: 31-37, 1980.
8. **Cadenas E, Boveris A, Ragan CI and Stoppani AO**. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Arch Biochem Biophys* 180: 248-257, 1977.
9. **Cancherini DV, Queliconi BB and Kowaltowski AJ**. Pharmacological and physiological stimuli do not promote Ca^{2+}-sensitive K^+ channel activity in isolated heart mitochondria. *Cardiovasc Res* 73: 720-728, 2007.
10. **Chance B, Sies H and Boveris A**. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527-605, 1979.
11. **Chen Q, Camara AK, Stowe DF, Hoppel CL and Lesnefsky EJ**. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion. *Am J Physiol Cell Physiol* 292: C137-C147, 2007.

12. **Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL and Lesnefsky EJ**. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* 278: 36027-36031, 2003.
13. **Debska G, Kicinska A, Dobrucki J, Dworakowska B, Nurowska E, Skalska J, Dolowy K and Szewczyk A**. Large-conductance K^+ channel openers NS1619 and NS004 as inhibitors of mitochondrial function in glioma cells. *Biochem Pharmacol* 65: 1827-1834, 2003.
14. **Duchen MR**. Mitochondria in health and disease: perspectives on a new mitochondrial biology. *Mol Aspects Med* 25: 365-451, 2004.
15. **Garlid KD and Paucek P**. Mitochondrial potassium transport: the K^+ cycle. *Biochim Biophys Acta* 1606: 23-41, 2003.
16. **Heinen A, Camara AK, Aldakkak M, Rhodes SS, Riess ML and Stowe DF**. Mitochondrial Ca^{2+}-induced K^+ influx increases respiration and enhances ROS production while maintaining membrane potential. *Am J Physiol Cell Physiol* 292: C148-C156, 2007.
17. **Kaczorowski GJ, Knaus HG, Leonard RJ, McManus OB and Garcia ML**. High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J Bioenerg Biomembr* 28: 255-267, 1996.
18. **Lambert AJ and Brand MD**. Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J Biol Chem* 279: 39414-39420, 2004.
19. **Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J and Hoppel CL**. Mitochondrial dysfunction in cardiac disease: ischemia-reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33: 1065-1089, 2001.
20. **Liu Y, Fiskum G and Schubert D**. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* 80: 780-787, 2002.
21. **Loschen G, Flohe L and Chance B**. Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. *FEBS Lett* 18: 261-264, 1971.
22. **Lowell BB and Shulman GI**. Mitochondrial dysfunction and type 2 diabetes. *Science* 307: 384-387, 2005.
23. **Munujos P, Knaus HG, Kaczorowski GJ and Garcia ML**. Cross-linking of charybdotoxin to high-conductance calcium-activated potassium channels: identification of the covalently modified toxin residue. *Biochemistry* 34: 10771-10776, 1995.
24. **O'Rourke B**. Evidence for mitochondrial K^+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.
25. **Ohya S, Kuwata Y, Sakamoto K, Muraki K and Imaizumi Y**. Cardioprotective effects of estradiol include the activation of large-conductance Ca^{2+}-activated K^+ channels in cardiac mitochondria. *Am J Physiol Heart Circ Physiol* 289: H1635-H1642, 2005.
26. **Oldenburg O, Cohen MV and Downey JM**. Mitochondrial K_{ATP} channels in preconditioning. *J Mol Cell Cardiol* 35: 569-575, 2003.
27. **Olesen SP, Munch E, Moldt P and Drejer J**. Selective activation of Ca^{2+}-dependent K^+ channels by novel benzimidazolone. *Eur J Pharmacol* 251: 53-59, 1994.
28. **Rego AC and Oliveira CR**. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem Res* 28: 1563-1574, 2003.
29. **Riess ML, Eells JT, Kevin LG, Camara AK, Henry MM and Stowe DF**. Attenuation of mitochondrial respiration by sevoflurane in isolated cardiac mitochondria is mediated in part by reactive oxygen species. *Anesthesiology* 100: 498-505, 2004.
30. **Riess ML, Kevin LG, McCormick J, Jiang MT, Rhodes SS and Stowe DF**. Anesthetic preconditioning: the role of free radicals in sevoflurane-induced attenuation of mitochondrial electron transport in Guinea pig isolated hearts. *Anesth Analg* 100: 46-53, 2005.

31. **Sato T, Saito T, Saegusa N and Nakaya H**. Mitochondrial Ca^{2+}-activated K^+ channels in cardiac myocytes: a mechanism of the cardioprotective effect and modulation by protein kinase A. *Circulation* 111: 198-203, 2005.
32. **Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG and Jiang MT**. Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 290: H434-H440, 2006.
33. **Szewczyk A, Skalska J, Glab M, Kulawiak B, Malinska D, Koszela-Piotrowska I and Kunz WS**. Mitochondrial potassium channels: from pharmacology to function. *Biochim Biophys Acta* 1757: 715-720, 2006.
34. **Turrens JF**. Mitochondrial formation of reactive oxygen species. *J Physiol* 552: 335-344, 2003.
35. **Turrens JF, Alexandre A and Lehninger AL**. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 237: 408-414, 1985.
36. **Turrens JF and Boveris A**. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 191: 421-427, 1980.
37. **Votyakova TV and Reynolds IJ**. DeltaPsi(m)-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* 79: 266-277, 2001.
38. **Wallner M, Meera P, Ottolia M, Kaczorowski GJ, Latorre R, Garcia ML, Stefani E and Toro L**. Characterization of and modulation by a beta-subunit of a human maxi K_{Ca} channel cloned from myometrium. *Receptors Channels* 3: 185-199, 1995.
39. **Wu SN**. Large-conductance Ca^{2+}- activated K^+ channels:physiological role and pharmacology. *Curr Med Chem* 10: 649-661, 2003.
40. **Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A and O'Rourke B**. Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* 298: 1029-1033, 2002.
41. **Yoshida T, Maulik N, Engelman RM, Ho YS and Das DK**. Targeted disruption of the mouse Sod I gene makes the hearts vulnerable to ischemic reperfusion injury. *Circ Res* 86: 264-269, 2000.

Chapter 4

The regulation of mitochondrial respiration by opening mK_{Ca} channels is age-dependent

André Heinen, Adrian Winning, Wolfgang Schlack, Markus W. Hollmann, Benedikt Preckel, Jan Fräβdorf, Nina C. Weber

European Journal of Pharmacology
2008 Jan;578(2):108-113

ABSTRACT

The protective potency of ischemic preconditioning decreases with increasing age. A key step in ischemic preconditioning is the opening of mitochondrial Ca^{2+} sensitive K^+ (mK_{Ca}) channels, which causes mild uncoupling of mitochondrial respiration. We hypothesized that aging reduces the effects of mK_{Ca} channel opening on mitochondrial respiration.

We measured the effects of mK_{Ca} channel opener NS1619 (30 μM) on mitochondrial respiration in isolated heart mitochondria from young (2-3 months) and old (22-26 months) Wistar rats. Oxygen consumption was monitored online after addition of 250 μM ADP (state 3 respiration), and after complete phosphorylation of ADP to ATP (state 4 respiration) in the presence or absence of the mK_{Ca} channel blocker paxilline (5 μM). The respiratory control index (RCI) was calculated as state 3 / state 4.

In mitochondria from young rats, NS1619 increased state 4 respiration by 11.9±4.1% (mean±S.E.M.), decreased state 3 respiration by 7.6±2.5%, and reduced the RCI from 2.6±0.03 (control) to 2.1±0.06 (all $P < 0.05$, $n = 12$ for all groups). Paxilline blocked the effect of NS1619 on state 4 respiration (0.7±2.8%), but did not affect the decrease in state 3 respiration; paxilline blunted the decrease of RCI. In mitochondria from old rats, NS1619 had neither effect on state 4 (0.4±1.6%), and state 3 respiration (-7.4±1.5%), nor on RCI (3.0±0.13 vs. 3.2±0.11, n=12).

Increasing age reduced the effects of mK_{Ca} opening on mitochondrial respiration. This might be one underlying reason of the decreased protective potency of ischemic preconditioning in the aged myocardium.

INTRODUCTION

The worldwide population of persons aged ≥ 65 years will increase from 420 million in 2000 to about 973 million in 2030. An increasing lifespan, expected to extend 10 years by 2050, is associated with an increase in the incidence and prevalence of chronic diseases such as coronary artery disease, which is a major cause for myocardial infarction. (22) The morbidity and mortality of myocardial infarction is enhanced with increasing age; (11) possibly due to an aging related loss of the protective potency of cardioprotective strategies, e.g. ischemic preconditioning. (20)

Ischemic preconditioning is a cardioprotective phenomenon by which short periods of ischemia reduce the deleterious consequences of a subsequent prolonged period of ischemia/reperfusion of the heart. (30) So far, most studies that investigated the protective effects and the underlying mechanism of ischemic preconditioning were conducted in young animals. There is strong evidence from the literature that the cardioprotective effect of ischemic preconditioning decreases with increasing age both in animals (1; 41) and in humans. (23) Lee et al. demonstrated a loss of protection by ischemic preconditioning in elderly patients undergoing coronary angioplasty. (23) A prolonged period of ischemia and the mitochondrial ATP-sensitive potassium (mK_{ATP}) channel activator nicorandil were able to (re)initiate a preconditioning state in these patients. The authors concluded that the impaired preconditioning response must result from some defects in the signal transduction of K^+ channel activation of the aged myocardium.

It is proposed that signalling pathways in preconditioning converge on the mitochondria. (28) Many reports strongly support the hypothesis that regulation of mitochondrial function by activation of K^+ channels in the inner mitochondrial membrane resulting in an increased K^+ influx into the mitochondrial matrix is a key step in the signal transduction cascade of ischemic preconditioning. (29; 31)

In addition to the importance of mK_{ATP} channels, there is increasing evidence for a role of Ca^{2+} sensitive potassium (K_{Ca}) channel opening in ischemic preconditioning. Recently, Cao et al. (6) demonstrated in isolated perfused rat hearts that blockade of K_{Ca} channels by paxilline abolished the reduction of infarct size caused by ischemic preconditioning. Furthermore, pharmacological preconditioning was initiated by the K_{Ca} channel activator 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619). Xu et al. (43) detected mitochondrial (m) K_{Ca} channels in the inner mitochondrial membrane of guinea pig ventricular cells and suggested a role for mK_{Ca} channels in protection against ischemic injury.

We aimed to investigate the effect of age-dependent changes in mK_{Ca} channel activation on mitochondrial respiration, and analyzed the effects of the mK_{Ca} channel agonist NS1619 and the antagonist Paxilline on mitochondrial respiration and oxidative phosphorylation in mitochondria isolated from young and old rat hearts.

MATERIALS AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and approved by the Institutional Committee for Animal Care and Use (Heinrich-Heine-University Düsseldorf, Germany).

Chemicals and reagents

KCl was purchased from EMD Chemicals (Gibbstown, NJ); all other chemicals were purchased from Sigma Chemical Co. (Taufkirchen, Germany). NS1619 and paxilline were dissolved in DMSO before they were added to the experimental buffer.

Mitochondrial isolation

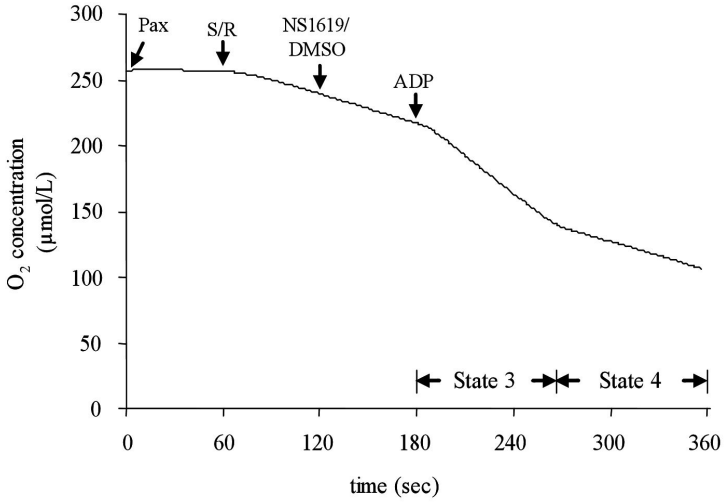
Heart mitochondria were isolated from young (2-3 months) and old (22-26 months) Wistar rats. Animals were anesthetized by an intraperitoneal injection of S(+)-ketamine (150 mg/kg). After decapitation, hearts were excised and heart mitochondria were isolated by differential centrifugation as described previously. (17; 18; 34) Briefly, atria were removed and ventricles were placed in isolation buffer [200 mmol/L mannitol, 50 mmol/L sucrose, 5 mmol/L KH_2PO_4, 5 mmol/L 3-(n-morpholino) propanesulfonic acid (MOPS), 1 mmol/L Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1% bovine serum albumin (BSA), pH 7.15 adjusted with KOH], and minced into 1 mm^3 pieces. The suspension was homogenized for 15 sec in 2.5 ml isolation buffer containing 5 U/ml protease (from Bacillus licheniformis, Enzyme Commission Number 3.4.21.14) , and for another 15 sec after addition of 17 ml isolation buffer. The suspension was centrifuged at 3220*g* for 10 min, the supernatant was removed, and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 3220*g* for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. Protein content was determined by the Bradford method. (4) All isolation procedures were conducted at 4°C.

Mitochondrial O_2 consumption

Oxygen consumption was measured polarographically at 27°C using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.25 mg protein/ml) were suspended in respiration buffer containing 130 mmol/L KCl, 5 mmol/L K_2HPO_4, 20 mmol/L MOPS, 2.5 mmol/L EGTA, 1 µmol/L $Na_4P_2O_7$, 0.1% BSA, pH 7.15 adjusted with KOH.

Mitochondrial respiration was initiated by administration of 10 mmol/L complex II substrate succinate (+10 µmol/L complex I blocker rotenone) after 60 sec (Fig. 1) in the presence or absence of 5 µmol/L mK_{Ca} channel blocker paxilline (Pax). The mK_{Ca} channel activator NS1619 (20, 30, or 50 µmol/L) or its vehicle DMSO (0.3%) were injected into the respiration chamber after 120 sec. State 3 respiration was initiated after 180 sec by addition of 250 µmol/L adenosine-diphosphate (ADP). Respiration rates were recorded under state 3 conditions and after complete phosphorylation of ADP to adenosine-triphosphate (ATP) (State 4).

Figure 1:



Representative traces of mitochondrial respiration experiments. Mitochondrial respiration was initiated by addition of 10 mmol/L succinate + 10µmol/L rotenone (S/R); State 3 respiration was initiated by addition of 250 µmol/L ADP. NS1619 or its vehicle DMSO (0.3 %) was administered at 120 sec. The respiration rates were analyzed both under state 3 conditions and after complete phosphorylation of ADP to ATP (State 4). When the effect of mK_{Ca} channel blockade by paxilline (Pax) was investigated, 5 µmol/L Pax were present during the whole experimental protocol.

The respiratory control index (RCI, state 3/state 4) and the P/O ratio (phosphate incorporated into ATP to oxygen consumed) were calculated as parameter of mitochondrial coupling between respiration and oxidative phosphorylation, and mitochondrial efficiency, respectively. From each heart, respiration measurements were repeated in 2 to 3 mitochondrial samples and the average was taken (and counted as n=1). Respiration rates are expressed as absolute rates in nmol O_2/mg/min or as percent of control.

To investigate concentration-dependent effects of mK_{Ca} channel opening on mitochondrial bioenergetics, we measured in a first series of experiments mitochondrial respiration in the absence (control) or presence of 20, 30, or 50 µmol/L NS1619 (NS20, NS30, or NS50, respectively).

To test if the effects of NS1619 were caused by mK_{Ca} channel opening, we added in a second series of experiments 5 µmol/L mK_{Ca} channel blocker Pax in the absence or presence of 30 µmol/L NS1619.

Statistical analysis

To analyse concentration-dependent effects of NS1619 (experimental series 1), group data were compared by analysis of variance, followed by Dunett's post hoc test (all vs. control).

To compare if the effects of NS30 were caused by mK_{Ca} channel opening (experimental series 2), group data were compared by analysis of variance, followed by Tukey's post hoc test. Data were considered statistically significant when $P<0.05$ and are presented as means±S.E.M.

RESULTS

Effect of aging on mitochondrial respiration

The respiration rates of isolated mitochondria from old rat hearts are reduced compared to mitochondria from young rat hearts both under "resting" state 4 conditions (72.5±6.3 nmol O_2/mg/min vs. 100.5±5.4 nmol O_2/mg/min) and "stimulated" state 3 conditions (218.4±13.9 nmol O_2/mg/min vs. 260.8±13.8 nmol O_2/mg/min) (Table 1). These age dependent changes in respiration rates resulted in an increased RCI in old rats compared to young rats (3.1±0.08 vs. 2.6±0.04). Aging did not affect the efficiency of oxidative phosporylation as demonstrated by no change in P/O ratio.

Table 1: Respiration rates under control conditions

	young	old
State 4 (nmol O_2/mg/min)	100.5±5.4	72.5±6.3 [a]
State 3 (nmol O_2/mg/min)	260.8±13.8	218.4±13.9 [a]
RCI (State 3/State 4)	2.6±0.04	3.1±0.08 [a]
P/O ratio	1.40±0.03	1.45±0.04

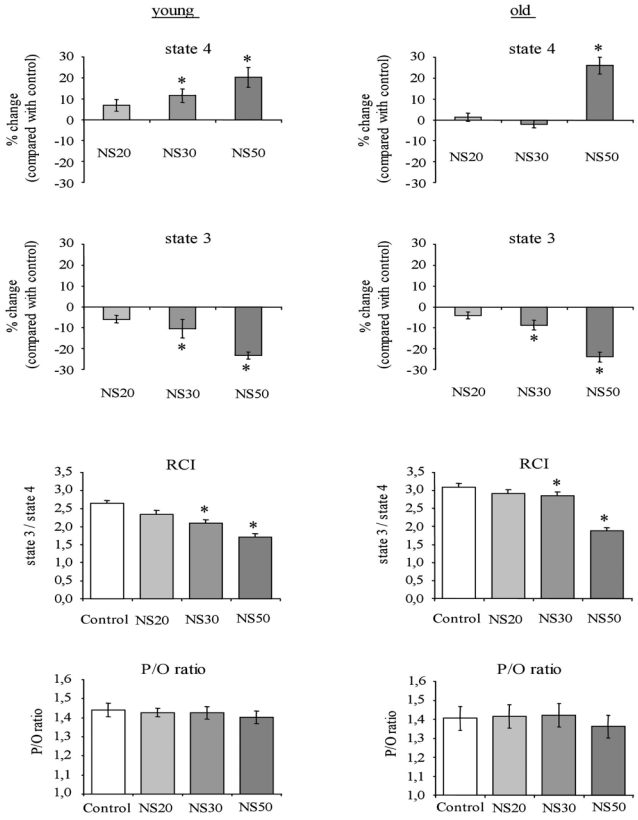
Data are mean±S.E.M.; [a]*P*<0.05 vs. young. RCI = respiratory control index (state 3/state 4). P/O ratio = ratio between phosphate incorporated into ATP to atoms O_2 consumed.

Concentration effects

The concentration-dependent effects of mK_{Ca} channel opening by NS1619 on mitochondrial bioenergetics were measured in our first series of experiments. Mitochondrial respiration was measured in the absence (control) or presence of 20, 30, or 50 µmol/L NS1619 (NS20, NS30, or NS50, respectively) (Fig. 2). We detected an age-dependent difference in respiration rates under state 4 conditions. In young rats, NS1619 increased oxygen consumption state 4 in a dose - dependent manner (NS20: 6.9±2.8%, P=ns; NS30: 11.5±3.2%, P<0.05; NS50: 20.3±4.8%, P<0.05).

In old rats, only the highest concentration of 50 µmol/L NS1619 increased state 4 respiration (NS50: 26.0±4.0%, P<0.05). Furthermore, NS1619 decreased state 3 respiration (NS20: -5.9±1.8%, P=ns; NS30: -10.5±4.4%, P<0.05; NS50: -23.3±1.8%, P<0.05) in young rats. In old rats, state 3 was decreased by NS30 (-8.7±2.4%, P<0.05) and NS50 (-24.0±2.3%, P<0.05). The respiratory control index, a parameter of the coupling between mitochondrial respiration and oxidative phosphorylation, was decreased by NS30, and NS50 both in young rats (-0.5±0.1, and -0.9±0.1, both P<0.05, respectively) and old rats, respectively (-0.3±0.1, and -1.0±0.1, both P<0.05). Opening of mK_{Ca} channel activation did not affect the efficiency of oxidative phosphorylation as shown by no changes in the P/O ratio neither in young nor in old rats.

Figure 2:



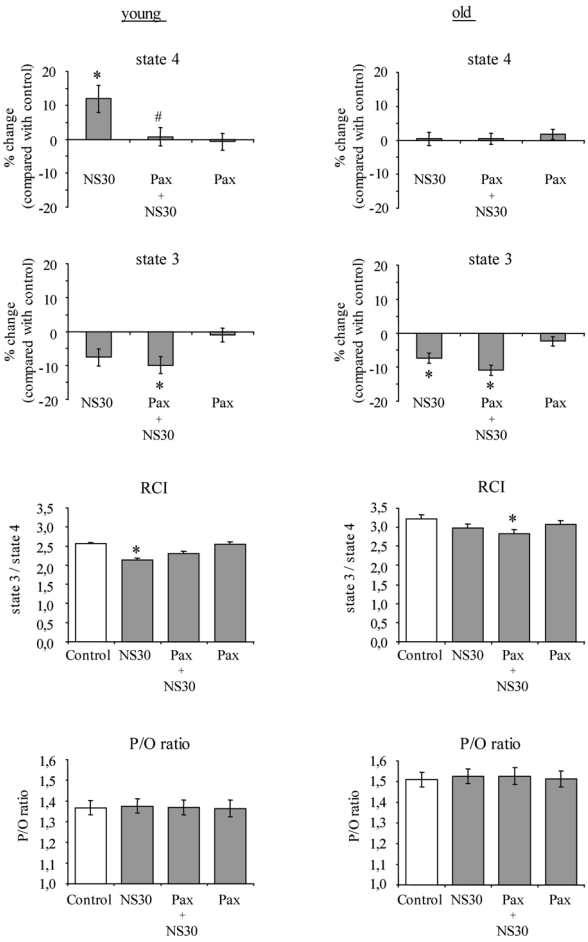
Summarized data for the concentration effects of 20, 30, or 50 µmol/L NS1619 (NS) on mitochondrial respiration in young (left panel, n=12 for all groups) or old (right panel, n=15 for all groups) rat heart mitochondria. Data are mean±S.E.M.; ANOVA followed by Dunnett's post hoc test; **P*<0.05 vs. control. RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into ATP and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation.

Blockade of mK_{Ca} channel activation

In the second series of experiments we tested by using mK_{Ca} channel blocker Pax, if the effects of NS1619 were caused by mK_{Ca} channel opening (Fig. 3). Pax alone had no effect on state 4 respiration, indicating mK_{Ca} channels were closed under the experimental conditions in both young (-0.7±2.5%, P=ns) and old rats (1.7±1.5%, P=ns). Pre-administration of Pax blocked the NS30-induced increase in state 4 respiration in young

rats (11.9±4.1% vs. 0.7±2.8%, P<0.05), but had no effect on state 4 respiration in the presence of NS30 in old rats (0.4±1.6%, P = ns). Pax did not affect state 3 respiration neither in young (-1.0±2.0%, P=ns) nor in old rats (-2.4±1.4%, P=ns), and did not reduce the NS30 induced decrease in state 3 respiration in both young (-9.9±2.5% vs. -7.6±2.5%, P=ns) and old rats (-10.9±1.5% vs. -7.4±1.5%, P=ns).

Figure 3:



Summarized data of the effects of 30 µmol/L NS1619 (NS30) on mitochondrial respiration and the blocking effects of mK_{Ca} channel antagonist paxilline (5 µmol/L, Pax) on mitochondrial respiration in young (left panel, n=12 for all groups) or old (right panel, n=12 for all groups) rat heart mitochondria. Data are mean±S.E.M., ANOVA followed by Tukey's post hoc test; $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs.

NS1619. RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into ATP and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation.

The NS30 induced decrease in RCI in young (-0.4±0.1 vs. -0.3±0.1, P=ns) and old rats (-0.3±0.2 vs. -0.1±0.2, P=ns) was not affected by Pax. Furthermore, blockade of mK_{Ca} channels did not affect the efficiency of oxidative phosphorylation as demonstrated by no changes in the P/O ratio neither in young nor in old rats.

DISCUSSION

The major findings of this study are that a) mitochondrial respiration is depressed in mitochondria from aged rat hearts in comparison with those from young hearts, and b) the regulation of mitochondrial respiration by opening of mK_{Ca} channels is age-dependent.

Mitochondria and alterations in mitochondrial function are deeply involved in the aging process. (24) Investigations on age-dependent changes in mitochondrial bioenergetics have produced conflicting results, showing significant changes (7; 8; 16) or no differences.(40) (for review see (25)) Fannin et al. demonstrated that these conflicting results can be explained by the finding that aging selectively decreases oxydative capacity in interfibrillar mitochondria, while respiration rates of subsarcolemmal mitochondria remaines unchanged. (12) Palmer et al. showed that a brief exposure to protease during the isolation procedure is requiered to isolate interfibrillar mitochondria. (33) In this study, we investigated respiration rates of interfibrillar mitochondria (or a mixed population), and confirmed that in this mitochondrial population the respiratory capacity of mitochondria from old rat hearts is reduced compared to mitochondria from young rat hearts.

Mitochondrial respiration and oxidative phosphorylation can be regulated by activation of K^+ channels in the inner mitochondrial membrane. It was suggested that activation of mitochondrial K^+ channels causes potassium influx from the intermembrane space into the mitochondrial matrix. Several recent studies have demonstrated a stimulating effect of matrix K^+ influx through mK_{ATP} channels on mitochondrial respiration. (10; 19; 27) Furthermore, there is strong evidence for the existence of another class of ion channels in the IMM that promote K^+ influx into the mitochondrial matrix: the Ca^{2+} dependent K^+ channel (mK_{Ca}). Siemen et al. (37) first reported mK_{Ca} channels in the IMM of glial cells. Xu et al. (43) very recently discovered these channels in cardiac myocyte mitochondria. Patch-clamp recordings from mitoplasts of these cells showed Ca^{2+} dependent, large K^+ conductance channels in the IMM and immunoblots of cardiac mitochondria with antibodies against the C terminal part of K_{Ca} channel identified a 55 kDa protein as part of

this putative channel. (32) O'Rourke suggested that mitochondrial K^+ channels function as energy dissipating channels (energy stored as the proton gradient, $\Delta\mu H$) by expending $\Delta\mu H$, in part to eject K^+ via an electroneutral K^+/H^+ exchanger. The resulting decrease in $\Delta\mu H$ in turn enhances electron flow. The bioenergetic consequence of K^+ channel opening would be accelerated cycling of K^+ ions between the matrix and the intermembrane space (i.e. matrix K^+ inflow through K^+ channel, K^+ extrusion via K^+/H^+ exchanger) and an increase in mitochondrial respiration. (31)

The regulation of mitochondrial function by mitochondrial K^+ channel activation is a key step to trigger ischemic and pharmacological preconditioning. (31; 42) It was shown that pharmacological blockade of K^+ channel abrogates the cardioprotective effects of ischemic preconditioning. (2; 6; 36) Furthermore, a preconditioning effect can be mimicked by administration of a K^+ channel opener. (6; 14; 26) The exact mechanism by which K^+ channel opening triggers and/or mediates preconditioning is incompletely understood. Most studies investigating the age-dependent effect of ischemic or anesthetic preconditioning found that the protective potency of this phenomenon is diminished or abolished in the aged heart. (1; 13; 20; 38; 41) Furthermore, Lee et al. demonstrated that preconditioning significantly enhances the tolerance of the heart to subsequent ischemia in adult but not in senescent patients. (23) Since a prolonged period of ischemia and the mK_{ATP} channel activator nicorandil were able to (re)initiate a preconditioning state, the authors concluded that the impaired preconditioning response is due to some defects in signal transduction of activation of K^+ channels in the aged heart. For this loss of efficiency of preconditioning in the aged heart, age-dependent alterations in the regulation of mitochondrial function by ion cycling might be a possible reason.

The aim of this study was to investigate, whether the bioenergetic consequences of mK_{Ca} channel opening by NS1619 on mitochondrial function are age-dependent. Here we show that opening of mK_{Ca} channels by NS1619 increases state 4 respiration only in young rat heart mitochondria and not in mitochondria isolated from old rat hearts. The finding that NS1619 accelerates mitochondrial respiration under resting conditions is in agreement with a previous study from Sato et al., who demonstrated a dose dependent increase in flavoprotein oxidation by mK_{Ca} channel activation. (35) Recently, Cancherini et al. described that NS1619 inhibited mitochondrial respiration. (5) The inhibitory effects of NS1619 on mitochondrial state 3 respiration were described before by Debska et al. (9) and our group (17; 18) and are confirmed by the present study. Cancherini et al. suggested that NS1619 promotes non-selective permeabilization of the inner mitochondrial membrane to ions. (5) In a previous study, (18) we discovered in isolated guinea pig heart mitochondria

that opening of mK_{Ca} channels by NS1619 accelerated mitochondrial state 4 respiration while maintaining mitochondrial membrane potential (ψ_m); conditions that were capable to increase generation of reactive oxygen species (ROS), a key trigger of preconditioning. (3; 18; 39) In the present study, mK_{Ca} channel activation also increased state 4 respiration in a dose dependent manner in young rat heart mitochondria. Furthermore, the effect of 30 µmol/L NS1619 was completely reversible by paxilline. We conclude that the effect of NS1619 on state 4 respiration is mK_{Ca} channel mediated. Furthermore, our results demonstrate for the first time that this effect is age dependent, since NS1619 had no effect on state 4 respiration in old rat heart mitochondria.

It is interesting to note that activation of mK_{Ca} channels by NS1619 had no effect on the efficiency of oxydative phosphorylation as seen by no change in P/O ratios while state 4 respiration was increased. A possible explanation for this observation is that K^+ channel opening regulates mitochondrial metabolism due to regulation of the matrix volume. It is proposed that the mitochondrial matrix contracts under state 3 conditions due to a reduced mitochondrial membrane potential, which is the driving force for cation and water uptake. (21) Opening of K^+ channels may reverse this matrix contracture to preserve oxydative phosphorylation. (15; 21)

From the results of this study it is hard to conclude whether a decreased density of mK_{Ca} channels in the inner mitochondrial membrane or a reduced sensitivity (or a combination) is responsible for the reduced effect of NS1619 on mitochondrial respiration. It is interesting to note that the high concentration of NS1619 accelerated state 4 respiration in young and old heart mitochondria in a comparable magnitude (approx. 20 nmol O_2/mg/min). This finding supports the possibility that a decreased sensitivity of mK_{Ca} channels causes the age-dependent difference in the effect of 30 µmol/L NS1619 (a concentration that has been shown to induce cardioprotection in young hearts).

We demonstrated recently that mK_{Ca} channel opening by NS1619 increased state 4 respiration independent if complex I substrate pyruvate or complex II substrate succinate (with or without rotenone) was used. (17; 18) Based on this finding, we conducted all experiments in isolated mitochondria respiring on complex II substrate succinate (+ rotenone). Nevertheless, this is a limitation of the present study.

From the observation of this study that the bioenergetic consequences of mK_{Ca} channel opening on mitochondrial respiration are age-dependent, we speculate that the aging related reduction in mK_{Ca} channel activation and the resulting effects on mitochondrial function might contribute to the decreased protective potency of ischemic preconditioning in the aged myocardium. Whether NS1619 does indeed not confer protection to the older hearts

against ischemia-reperfusion injury needs further investigation in a functional correlate study.

ACKNOWLEDGEMENTS

The authors wish to thank Matthias L. Riess, Amadou K.S. Camara, David F. Stowe and Mohammed Aldakkak for their support with this study.

REFERENCES

1. **Abete P, Ferrara N, Cioppa A, Ferrara P, Bianco S, Calabrese C, Cacciatore F, Longobardi G and Rengo F**. Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiol* 27: 1777-1786, 1996.
2. **Auchampach JA, Grover GJ and Gross GJ**. Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res* 26: 1054-1062, 1992.
3. **Becker LB**. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc Res* 61: 461-470, 2004.
4. **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
5. **Cancherini DV, Queliconi BB and Kowaltowski AJ**. Pharmacological and physiological stimuli do not promote Ca^{2+}-sensitive K^+ channel activity in isolated heart mitochondria. *Cardiovasc Res* 73: 720-728, 2007.
6. **Cao CM, Xia Q, Gao Q, Chen M and Wong TM**. Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning. *J Pharmacol Exp Ther* 312: 644-650, 2005.
7. **Chen JC, Warshaw JB and Sanadi DR**. Regulation of mitochondrial respiration in senescence. *J Cell Physiol* 80: 141-148, 1972.
8. **Chiu YJ and Richardson A**. Effect of age on the function of mitochondria isolated from brain and heart tissue. *Exp Gerontol* 15: 511-517, 1980.
9. **Debska G, Kicinska A, Dobrucki J, Dworakowska B, Nurowska E, Skalska J, Dolowy K and Szewczyk A**. Large-conductance K^+ channel openers NS1619 and NS004 as inhibitors of mitochondrial function in glioma cells. *Biochem Pharmacol* 65: 1827-1834, 2003.
10. **Debska G, Kicinska A, Skalska J, Szewczyk A, May R, Elger CE and Kunz WS**. Opening of potassium channels modulates mitochondrial function in rat skeletal muscle. *Biochim Biophys Acta* 1556: 97-105, 2002.
11. **Devlin W, Cragg D, Jacks M, Friedman H, O'Neill W and Grines C**. Comparison of outcome in patients with acute myocardial infarction aged > 75 years with that in younger patients. *Am J Cardiol* 75: 573-576, 1995.
12. **Fannin SW, Lesnefsky EJ, Slabe TJ, Hassan MO and Hoppel CL**. Aging selectively decreases oxidative capacity in rat heart interfibrillar mitochondria. *Arch Biochem Biophys* 372: 399-407, 1999.
13. **Fenton RA, Dickson EW, Meyer TE and Dobson JG, Jr.** Aging reduces the cardioprotective effect of ischemic preconditioning in the rat heart. *J Mol Cell Cardiol* 32: 1371-1375, 2000.
14. **Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA and Grover GJ**. Cardioprotective effect of diazoxide and its

interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. *Circ Res* 81: 1072-1082, 1997.

15. **Halestrap AP**. The regulation of the matrix volume of mammalian mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism. *Biochim Biophys Acta* 973: 355-382, 1989.
16. **Hansford RG**. Lipid oxidation by heart mitochondria from young adult and senescent rats. *Biochem J* 170: 285-295, 1978.
17. **Heinen A, Aldakkak M, Stowe DF, Rhodes SS, Riess ML, Varadarajan SG and Camara AK**. Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca^{2+} sensitive K^+ channels. *Am J Physiol Heart Circ Physiol* [Epub ahead of print], 2007.
18. **Heinen A, Camara AK, Aldakkak M, Rhodes SS, Riess ML and Stowe DF**. Mitochondrial Ca^{2+}-induced K^+ influx increases respiration and enhances ROS production while maintaining membrane potential. *Am J Physiol Cell Physiol* 292: C148-C156, 2007.
19. **Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A and Terzic A**. Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. *Am J Physiol* 275: H1567-H1576, 1998.
20. **Juhaszova M, Rabuel C, Zorov DB, Lakatta EG and Sollott SJ**. Protection in the aged heart: preventing the heart-break of old age? *Cardiovasc Res* 66: 233-244, 2005.
21. **Kowaltowski AJ, Seetharaman S, Paucek P and Garlid KD**. Bioenergetic consequences of opening the ATP-sensitive K^+ channel of heart mitochondria. *Am J Physiol Heart Circ Physiol* 280: H649-H657, 2001.
22. **Lakatta EG and Levy D**. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. *Circulation* 107: 346-354, 2003.
23. **Lee TM, Su SF, Chou TF, Lee YT and Tsai CH**. Loss of preconditioning by attenuated activation of myocardial ATP-sensitive potassium channels in elderly patients undergoing coronary angioplasty. *Circulation* 105: 334-340, 2002.
24. **Lenaz G**. Role of mitochondria in oxidative stress and ageing. *Biochim Biophys Acta* 1366: 53-67, 1998.
25. **Lesnefsky EJ and Hoppel CL**. Oxidative phosphorylation and aging. *Ageing Res Rev* 5: 402-433, 2006.
26. **Loubani M, Fowler A, Standen NB and Galinanes M**. The effect of gliclazide and glibenclamide on preconditioning of the human myocardium. *Eur J Pharmacol* 515: 142-149, 2005.
27. **Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DM and Sack MN**. Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res* 89: 787-792, 2001.
28. **Murphy E**. Primary and secondary signaling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. *Circ Res* 94: 7-16, 2004.
29. **Murphy E and Steenbergen C**. Preconditioning: The mitochondrial connection. *Annu Rev Physiol* 69: 51-67, 2007.
30. **Murry CE, Jennings RB and Reimer KA**. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
31. **O'Rourke B**. Evidence for mitochondrial K^+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.
32. **Ohya S, Kuwata Y, Sakamoto K, Muraki K and Imaizumi Y**. Cardioprotective effects of estradiol include the activation of large-conductance Ca^{2+}-activated K^+ channels in cardiac mitochondria. *Am J Physiol Heart Circ Physiol* 289: H1635-H1642, 2005.

33. **Palmer JW, Tandler B and Hoppel CL**. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem* 252: 8731-8739, 1977.
34. **Riess ML, Eells JT, Kevin LG, Camara AK, Henry MM and Stowe DF**. Attenuation of mitochondrial respiration by sevoflurane in isolated cardiac mitochondria is mediated in part by reactive oxygen species. *Anesthesiology* 100: 498-505, 2004.
35. **Sato T, Saito T, Saegusa N and Nakaya H**. Mitochondrial Ca^{2+}-activated K^+ channels in cardiac myocytes: a mechanism of the cardioprotective effect and modulation by protein kinase A. *Circulation* 111: 198-203, 2005.
36. **Shintani Y, Node K, Asanuma H, Sanada S, Takashima S, Asano Y, Liao Y, Fujita M, Hirata A, Shinozaki Y, Fukushima T, Nagamachi Y, Okuda H, Kim J, Tomoike H, Hori M and Kitakaze M**. Opening of Ca^{2+}-activated K^+ channels is involved in ischemic preconditioning in canine hearts. *J Mol Cell Cardiol* 37: 1213-1218, 2004.
37. **Siemen D, Loupatatzis C, Borecky J, Gulbins E and Lang F**. Ca^{2+}-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. *Biochem Biophys Res Commun* 257: 549-554, 1999.
38. **Sniecinski R and Liu H**. Reduced efficacy of volatile anesthetic preconditioning with advanced age in isolated rat myocardium. *Anesthesiology* 100: 589-597, 2004.
39. **Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG and Jiang MT**. Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 290: H434-H440, 2006.
40. **Takasawa M, Hayakawa M, Sugiyama S, Hattori K, Ito T and Ozawa T**. Age-associated damage in mitochondrial function in rat hearts. *Exp Gerontol* 28: 269-280, 1993.
41. **Tani M, Suganuma Y, Hasegawa H, Shinmura K, Ebihara Y, Hayashi Y, Guo X and Takayama M**. Decrease in ischemic tolerance with aging in isolated perfused Fischer 344 rat hearts: relation to increases in intracellular Na^+ after ischemia. *J Mol Cell Cardiol* 29: 3081-3089, 1997.
42. **Weber NC, Toma O, Damla H, Wolter JI, Schlack W and Preckel B**. Upstream signaling of protein kinase C-epsilon in xenon-induced pharmacological preconditioning. Implication of mitochondrial adenosine triphosphate dependent potassium channels and phosphatidylinositol-dependent kinase-1. *Eur J Pharmacol* 539: 1-9, 2006.
43. **Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A and O'Rourke B**. Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* 298: 1029-1033, 2002.

Chapter 5

Helium-induced preconditioning in young and old rat heart - Impact of mK_{Ca} channel activation

[1] André Heinen, [1] Ragnar Huhn, Kirsten M. A. Smeele, Coert J. Zuurbier, Wolfgang Schlack, Benedikt Preckel, Nina C. Weber, Markus W. Hollmann,

[1] These authors contributed equally to this work

Submitted for Publication

ABSTRACT

The noble gas helium induces cardiac preconditioning. It is unknown whether helium-preconditioning is mediated by activation of mitochondrial K^+ channels. We investigated if helium preconditioning is 1) mediated by activation of K_{Ca} channels, 2) results in mitochondrial uncoupling, and 3) whether helium-preconditioning is age-dependent. Anesthetized Wistar rats were assigned to one of six groups (each n = 10). Young (2-3 months) and aged (22-24 months) control animals were not further treated (Con and Age-Con). Preconditioning groups (He-PC and Age-He-PC) inhaled 70% helium for 3x5 min. The K_{Ca} blocker Iberiotoxin (Ibtx, 6 µg kg^{-1} min^{-1}) was administered in young animals, with and without helium (Ibtx+He-PC and Ibtx). Animals were exposed to 25 min regional myocardial ischemia followed by 120 min reperfusion, and infarct size was determined. In additional experiments, cardiac mitochondria were isolated and the respiratory control index (RCI) was determined as state 3 respiration / state 4 respiration. Helium reduced infarct size in young rats from 61±7 % to 36±14 % ($P<0.05$ vs. Con). Infarct size reduction was abolished by Iberiotoxin (60±11 %; $P<0.05$ vs. He-PC), whereas Ibtx alone had no effect (59±8 %; n.s. vs. Con). In aged animals Helium had no effect on infarct size (Age-Con: 59±7 % vs. Age-He-PC: 58±8 %, n.s.). Helium reduced the RCI in young rats (2.76±0.05 to 2.43±0.15, $P<0.05$) but not in aged animals (Age-Con: 2.87±0.17 vs. Age-He-PC: 2.87±0.07, n.s.). Ibtx abrogated the effect of helium on RCI (2.73±0.15, $P<0.05$ vs. He-PC), but had no effect on mitochondrial respiration alone (2.75±0.05; n.s. vs. Con). Helium causes mitochondrial uncoupling, and induces preconditioning in young rats via K_{Ca} channel activation. However, these effects are lost in aged rats.

INTRODUCTION

Ischemic heart disease, with its clinical consequences of acute myocardial infarction, sudden cardiac death, arrhythmias and heart failure is the leading cause of morbidity and mortality in industrialized nations. Several studies demonstrated tissue protective effects of PC during ischemia-reperfusion interventions, both in animals (15; 16; 23) and humans. (4; 5)

However, most of these studies were conducted in young and healthy animals. The morbidity and mortality of myocardial infarction is increased with increasing age, (6; 7; 19) possibly partly due to an aging related loss of the protective potency of cardioprotective strategies, e.g. preconditioning (PC). (1; 11; 12; 32) The underlying reason for this loss of cardioprotection in the senescent heart is unknown. Lee et al. (12) demonstrated a loss of protection in elderly patients (older than 65 years) undergoing coronary angioplasty compared to patients younger than 55 years. Since a prolonged period of ischemia and the mitochondrial ATP-sensitive potassium channel activator nicorandil were able to (re)initiate a preconditioning state in the older patients, the authors concluded that the impaired preconditioning response is caused by some defects in signal transduction of activation of ATP-sensitive potassium (K_{ATP}) channels with aging. There is evidence that regulation of mitochondrial function by activation of potassium (K^+) channels in the inner mitochondrial membrane with the consequence of K^+ influx into the mitochondrial matrix is a key step in the signal transduction cascade of PC. (22; 25) Recently, we discovered that the effect of activation of calcium sensitive potassium (K_{Ca}) channels on mitochondrial function is age dependent. (10) It was shown that activation of this channel is critically involved in the signal transduction pathway of PC. (30)

A recent study demonstrated that the noble gas helium is able to mimic the cardioprotective effect of PC. (28) Helium confers cardioprotection via modulation of the mitochondrial permeability transition pore (mPTP). (28) It is suggested that opening of the mPTP can be prevented by alterations in mitochondrial function. (8) However, it is unknown whether helium-induced preconditioning is mediated by K_{Ca} channels with the consequence of altered mitochondrial respiration, and whether helium initiates preconditioning in the senescent heart. Here, we hypothesize that helium-induced preconditioning 1) is mediated by activation of K_{Ca} channels, 2) results in mitochondrial uncoupling, and 3) is abolished in the aged myocardium.

MATERIALS AND METHODS

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was performed in accordance with the requirements of the Animal Ethics Committee of the University of Amsterdam.

Materials

Helium was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, the Netherlands). KCl was purchased from EMD Chemicals (Gibbstown, NJ); all other chemicals were purchased from Sigma Chemical Co. (Taufkirchen, Germany). The polyclonal K_{Ca} channel beta 1 subunit antibody and the immunizing peptide were purchased from Abcam (Cambridge, UK).

Surgical preparation and experimental protocol for infarct size determination

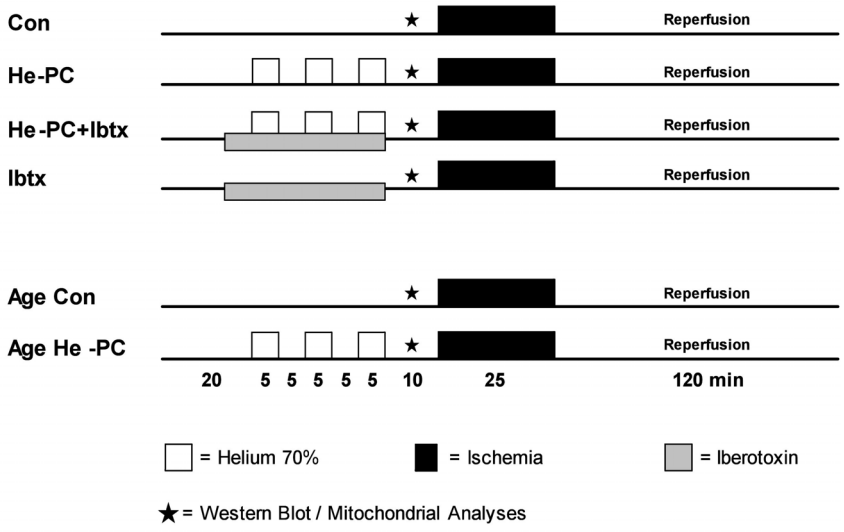
Animals had free access to food and water at all times before the start of the experiments. Young (3-4 months) male Hannover Wistar rats (352 ± 15 g) and old (22-24 months) male Hannover Wistar rats (621 ± 34 g) were anesthetized by intraperitoneal S-ketamine injection (150 mg/kg) and diazepam (1.5 mg/kg).

Surgical preparation was performed as described previously.(26; 33) In brief, after tracheal intubation, the lungs were ventilated, and respiratory rate was adjusted to maintain PCO_2 within physiological limits. Body temperature was maintained at 38°C by the use of a heating pad. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. Anesthesia was maintained by continuous α-chloralose infusion. A lateral left sided thoracotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery. All animals were left untreated for 20 minutes before the start of the respective experimental protocol. Aortic pressure was digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and was continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments).

Rats were divided into six groups (Fig. 1):

All animals underwent 25 min of coronary artery occlusion and 2 hours of reperfusion (I/R).

Figure 1: Experimental protocol



Control group (Con) (n = 10): After surgical preparation, rats received 30% oxygen plus 70% nitrogen.

Helium preconditioned group (He-PC) (n = 10): Rats received Helium 70% for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before I/R. The other 30% gas consisted of 30% oxygen.

Helium preconditioned group with Iberiotoxin (He-PC+Ibtx) (n = 10): Rats received Helium 70% for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before I/R. The other 30% gas consisted of 30% oxygen. Ibtx was administered continuously over a time period of 30 minutes starting 5 min prior to the first preconditioning stimulus.

Iberiotoxin group (Ibtx) (n = 10): Rats received Ibtx continuously over a time period of 30 minutes starting 5 min prior to the first preconditioning stimulus.

Aged control group (Age Con) (n = 10): After surgical preparation, rats received 30% oxygen plus 70% nitrogen.

Aged Helium preconditioned group (Age He-PC) (n = 10): Rats received Helium 70% for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before I/R. The other 30% gas consisted of 30% oxygen.

Infarct size measurement

After 120 minutes of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for perfusion with ice cold normal saline via the aortic root at a perfusion pressure of 80 cm H_2O in order to wash out intravascular blood. After 5 minutes of perfusion, the coronary artery was re-occluded and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 minutes. Intravascular Evans blue was then washed out by perfusion for 10 minutes with normal saline. This treatment identified the area at risk as unstained. The heart was then cut into transverse slices, 2 mm thick. The slices were stained with 0.75% triphenyltetrazolium chloride solution for 10 minutes at 37°C, and fixed in 4% formalin solution for 24 hours at room temperature. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL).

For mitochondrial respiration and Western Blot analysis additional experiments (each n = 8) were performed. Hearts were excised 5 min before the onset of ischemia (total baseline 50 min).

Mitochondrial isolation

Heart mitochondria were isolated by differential centrifugation as described previously.(10) Briefly, atria were removed and ventricles were placed in isolation buffer [200 mmol/L mannitol, 50 mmol/L sucrose, 5 mmol/L KH_2PO_4, 5 mmol/L 3-(n-morpholino) propanesulfonic acid (MOPS), 1 mmol/L Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1% bovine serum albumin (BSA), pH 7.15 adjusted with KOH], and minced into 1 mm^3 pieces. The suspension was homogenized for 15 sec in 2.5 ml isolation buffer containing 5 U/ml protease (from Bacillus licheniformis, Enzyme Commission Number 3.4.21.14), and for another 15 sec after addition of 17 ml isolation buffer. The suspension was centrifuged at 3220*g* for 10 min, the supernatant was removed, and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 3220*g* for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. Protein content was determined by the Bradford method. All isolation procedures were conducted at 4°C.

Mitochondrial respiration

Oxygen consumption was measured polarographically at 37°C using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.3 mg protein/ml) were suspended in respiration buffer containing 130 mmol/L KCl, 5 mmol/L K_2HPO_4, 20 mmol/L MOPS, 2.5 mmol/L EGTA, 1 µmol/L $Na_4P_2O_7$, 0.1% BSA, pH 7.15 adjusted with KOH. Mitochondrial respiration was initiated by administration of 10 mmol/L complex II substrate succinate (+10 µmol/L complex I blocker rotenone) after 60 sec. State 3 respiration was initiated after 120 sec by addition of 200 µmol/L adenosine-diphosphate (ADP). Respiration rates were recorded under state 3 conditions and after complete phosphorylation of ADP to adenosine-triphosphate (ATP) (State 4). The respiratory control index (RCI, state 3/state 4) and the P/O ratio (phosphate incorporated into ATP to oxygen consumed) were calculated as parameter of mitochondrial coupling between respiration and oxidative phosphorylation, and mitochondrial efficiency, respectively. From each heart, respiration measurements were repeated in 3 mitochondrial samples and the average was taken (and counted as n=1). Respiration rates are expressed as absolute rates in nmol O_2/mg/min.

Western blot analysis

The content of K_{Ca} channels in the mitochondria was determined by Western blot analysis. 100 µl of mitochondrial suspension was treated with 5 µl Triton X 100 (10%), 20 µl KCL (4.5 M), and protease inhibitor mix (aprotinin, leupeptin and pepstatin), stirred, and incubated at room temperature for 5 min. After centrifugation (10000 g, 5 min), the protein concentration was determined by the Lowry method. (18) Subsequently, equal amounts of mitochondrial protein (30 µg) were mixed with loading buffer (1:1) containing Tris-HCl, glycerol and bromphenol blue. Samples were loaded on a 12% SDS-PAGE gel, separated by electrophoresis and transferred to a PVDF membrane by tank blotting (100V, 2h). Unspecific binding of the antibody was blocked by incubation with 5% skimmed milk solution in Tris buffered saline containing Tween (TBS-T) for 2 hours. Subsequently, the membrane was incubated over night at 4°C with the K_{Ca} channel beta 1 subunit antibody (1:1000). After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands were visualized by chemiluminescence and detected on X-ray film (Hyperfilm ECL, Amersham) using the enhanced chemiluminescence system Santa Cruz. The blots were quantified using a Kodak Image Station® (Eastman Kodak Comp., Rochester, NY) and the results are presented as ratio of K_{Ca} beta1 subunit (arbitrary units)

to citrate synthase activity (mU/mg). Equal loading of protein on the gel was additionally proved by Coomassie blue staining of the gels. For identification of the specific K_{Ca} beta 1 subunit band, additional blocking experiments were conducted using the immunizing peptide in a large molar excess (~70 fold) for competitive inhibition of antibody-protein binding.

Determination of enzyme activities

Citrate synthase (CS) activity, a mitochondrial marker, was measured according to standard spectrophotometric procedures (2) and served as a control for Western blot results of mitochondrial K_{Ca} channels. It was shown that CS activity does not change with increasing age. (21; 29)

Statistical Analysis

Data are expressed as mean ± SD. Heart rate (HR, in bpm) and mean AOP (AOPmean, in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion period. Inter-group differences of hemodynamic data were analyzed (SPSS Science Software, version 12.0.1) by performing a One-way ANOVA followed by Tukey's post-hoc test. Time effects (changes from baseline value) during the experiments were analyzed by using a One-way ANOVA followed by Dunnett's post-hoc test. Infarct sizes were analyzed by a One-way ANOVA followed by Tukey's post-hoc test. Changes within and between groups were considered statistically significant if $p<0.05$. Mitochondrial respiration results and Western blot data were analyzed by a One-way ANOVA followed by Tukey's post-hoc test.

RESULTS

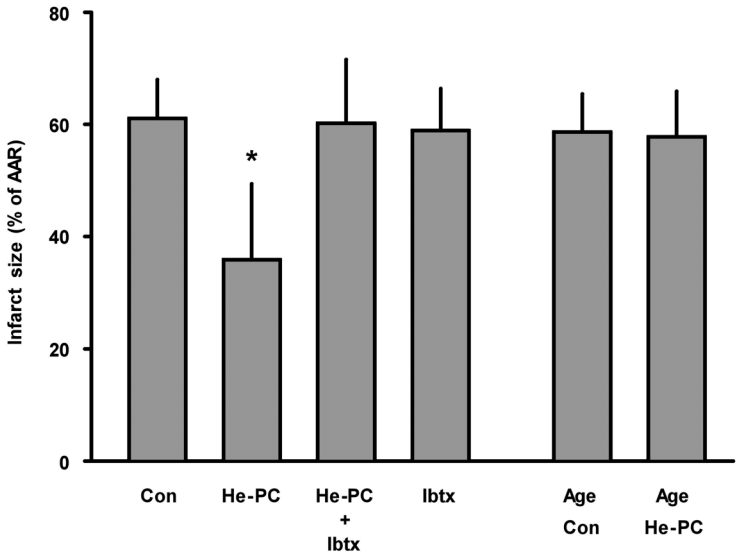
Infarct size measurement

Helium-induced preconditioning reduced infarct size in young animals from 61 ± 7 % in controls (n = 10) to 36 ± 14 % (n = 10, $P<0.05$, Fig. 2). Administration of Iberiotoxin during the preconditioning period (n = 10) completely abolished cardioprotection (60 ± 11 %; ns vs. Con). Iberiotoxin alone (n = 10) had no effect on infarct size (59 ± 8 %; ns vs. Con). Infarct size in aged controls (n = 10) was comparable to young controls (59 ± 7%). In contrast to young rats, Helium did not reduce infarct size in aged rats (58 ± 8 %, n = 10, ns vs. Age Con, Fig. 2).

Hemodynamic variables

Hemodynamic variables are summarized in table 1. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischemia or reperfusion. At the end of the experiments, mean aortic pressure and heart rate were significantly decreased compared with baseline in all groups.

Figure 2: Infarct size measurement



Histogram shows the infarct size (percent of area at risk, AAR) of controls (Con), preconditioning with 70% Helium (He-PC), preconditioning with 70% Helium combined with Iberiotoxin (He-PC+Ibtx), Iberiotoxin alone (Ibtx), controls in aged rats (Age Con) and preconditioning in aged rats with 70% Helium (Age He-PC). Data are presented as mean ± SD, *p < 0.05 vs. control group.

Mitochondrial function

The respiratory control indices are shown in figure 3. There was no significant difference in the RCI between young (n = 8) and aged (n = 8) control rats (2.76±0.05 vs. 2.87±0.10, ns). Helium preconditioning reduced the RCI in young rats (n = 8; 2.43±0.12, $p<0.05$ vs. Con), but had no effect on the RCI in aged rats (n = 8; 2.87±0.09, ns vs. Age Con). RCI reduction was completely abolished by administration of the mK_{Ca} channel blocker Ibtx (2.73±0.15, ns vs. Con), while Ibtx itself had no effect on RCI (2.75±0.05, ns vs. Con).

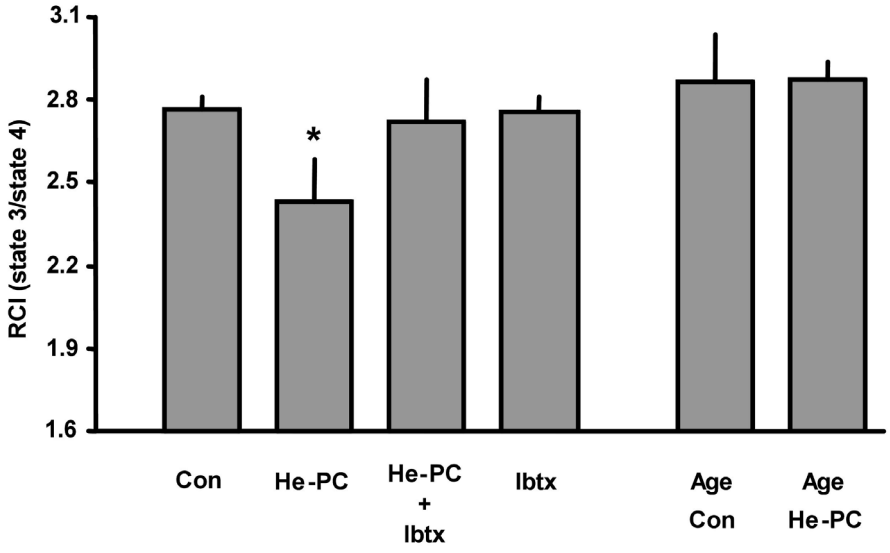
There was no difference between all groups in the efficiency of oxidative phosphorylation as demonstrated by no changes in the P/O ratio.

Table 1: Hemodynamic variables

	Baseline	Washout 3	Ischemia	Reperfusion	
			15	30	120
Heart rate (bpm)					
Con	445 ± 27	428 ± 35	424 ± 35	397 ± 36	369 ± 33*
He-PC	448 ± 12	443 ± 21	449 ± 27	397 ± 36*	374 ± 32*
He-PC+Ibtx	435 ± 29	417 ± 31	426 ± 28	390 ± 23*	361 ± 25*
Ibtx	461 ± 31	439 ± 27	459 ± 20	429 ± 27	385 ± 40*
Age Con	421 ± 28	405 ± 24	410 ± 28	374 ± 37*	323 ± 44*
Age He-PC	409 ± 27	407 ± 28	402 ± 29	367 ± 33*	334 ± 34*
Mean aortic pressure (mmHg)					
Con	127 ± 18	112 ± 20	101 ± 22	91 ± 23*	68 ± 11*
He-PC	133 ± 25	125 ± 23	108 ± 31	95 ± 23*	76 ± 19*
He-PC+Ibtx	117 ± 21	119 ± 17	101 ± 25	88 ± 21*	72 ± 9*
Ibtx	127 ± 24	129 ± 16	124 ± 16	94 ± 18*	65 ± 17*
Age Con	114 ± 26	114 ± 18	115 ± 24	98 ± 21	78 ± 21*
Age He-PC	119 ± 21	120 ± 20	111 ± 25	92 ± 16*	77 ± 19*

Data are Mean ± SD. Con = control group; Age = aged rats; He-PC = Helium preconditioning; Ibtx = Iberiotoxin. *P<0.05 vs. baseline.

Figure 3: Mitochondrial respiration



	Con	He-PC	He-PC + Ibtx	Ibtx	Age Con	Age He-PC
state 3 (nmol O_2/mg/min)	241±21	239±22	241±23	239±21	289±19	288±27
state 4 (nmol O_2/mg/min)	87±7	100±12	89±10	87±9	101±7	100±8
P/O ratio	1.7±0.05	1.7±0.05	1.7±0.07	1.7±0.05	1.8±0.06	1.8±0.06

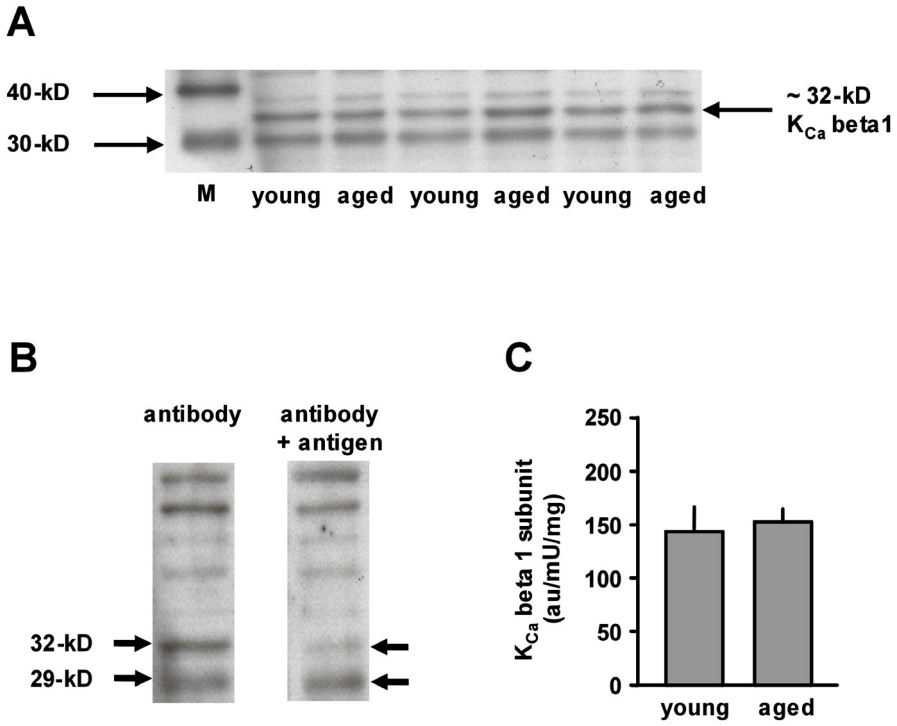
Summarized data for the effects of Helium-induced preconditioning on mitochondrial respiration. RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into adenosine-triphosphate and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation. Data are presented as mean ± SD, *p < 0.05 vs. control group.

Western blot analysis

Figure 4 (panel C) shows that there was no difference of K_{Ca} beta1 subunit expression in mitochondrial lysates from young and aged rat heart mitochondria (normalized to citrate synthase activity; young: 143 ± 23 au, old: 153 ± 12 au, n.s.).

The analysis of citrate synthase activity showed no difference between young and old mitochondria (young: 1012 ± 109 mU/mg, aged: 1065 ± 61 mU/mg, n.s.).

Figure 4: Western blot analysis



A) Representative Western blot (K_{Ca} channel beta 1 subunit) showing two major bands at ~32-kD and ~29-kD, respectively in mitochondrial lysate from both young and old aged heart mitochondria. B) Identification of specific band by immunizing peptide blocking experiment. The arrows denote positions of the 32-kD and the 29-kD bands. Blocking the antibody with the antigen demasks the specific band (32-kD) by strongly reducing the intensity of the band (right), while the intensity of the 29-kD band (and other non-specific bands) remains unchanged. C) Summarized data of the Western blot analysis of K_{Ca} channel beta 1 subunit normalized to citrate synthase activity.

DISCUSSION

The main findings of our study are that helium-induced preconditioning 1) is mediated by activation of K_{Ca} channels, 2) is accompanied by alterations in mitochondrial respiration, and 3) is abolished in the senescent heart.

In a recent study, the noble gas helium, a gas without anesthetic properties, was found to mimic the cardioprotective effect of preconditioning. (28) The results of the present study

are in line with these previous findings that helium confers cardioprotection *in vivo* as seen by a strong infarct size reduction in the helium preconditioning group compared with control hearts. (28) It was beyond the scope of the present study to unravel the complete mechanism of helium-induced preconditioning. However, our results demonstrate that activation of K_{Ca} channels is critically involved in the signal transduction pathway because the infarct size reducing effect of helium was completely abrogated by the K_{Ca} channel antagonist iberiotoxin. A central role of K_{Ca} channels in preconditioning has been shown by several studies demonstrating that either pharmacological activation of these channels initiates cardioprotection, or that pharmacological preconditioning can be blocked by K_{Ca} channel antagonists. (3; 27; 30; 31; 34) In 2002, Xu et al. reported not only evidence for the existence of K_{Ca} channels in the inner mitochondrial membrane of ventricular myocytes, the authors also demonstrated a cardioprotective potency of mitochondrial K_{Ca} (mK_{Ca}) channel activation. (34) Recently, we showed that activation of mK_{Ca} channels increases mitochondrial state 4 respiration and reduces the respiratory control index in isolated guinea pig heart mitochondria. (9) In the present study, helium-induced preconditioning did not only reduce infarct size, it also caused a significant reduction in the mitochondrial respiratory control index. Furthermore, helium-induced reduction in the respiratory control index was completely abolished by co-administration of iberiotoxin. We conclude from these data that helium confers cardioprotection by activation of mK_{Ca} channels with the consequence of mild mitochondrial uncoupling. A mild mitochondrial uncoupling during the trigger phase of preconditioning may represent a common characteristic of mitochondria in a "preconditioned" state. (13; 17; 20; 34)

The mechanism by which mK_{Ca} channel activation mediates cardioprotection is still incompletely understood. Opening of mK_{Ca} channels is capable to cause a slight increase in mitochondrial reactive oxygen species generation. (9) Stowe et al. (31) demonstrated that the cardioprotective effect of K_{Ca} channel agonist 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619) requires superoxide radical generation during the preconditioning stimulus. Furthermore, the authors demonstrated that preconditioning by NS1619 reduces mitochondrial calcium overload and mitochondrial reactive oxygen species production during the subsequent period of ischemia and early reperfusion. (31) Such a reduction in mitochondrial calcium overload and reactive oxygen species generation has been suggested to prevent mPTP opening. (8; 14) Pagel et al. demonstrated that the infarct size reducing effect of helium was abolished by co-administration of the mPTP opener atractyloside, thereby showing that modulation of the mPTP is involved in helium-induced preconditioning. (28)

In the present study, helium-induced preconditioning did not reduce infarct size in the aged rat heart. The underlying reason for the age-related loss of the cardioprotective potency of helium-induced preconditioning is yet unknown. Our results show that not only the infarct size reducing effect of helium is lost in the senescent rat heart, but that also the helium-induced effect on mitochondrial respiration before the onset of the lethal ischemia is abolished. Based on these data we suggest that the aging related blockade of helium-induced preconditioning is related to some defects at the level of the mK_{Ca} channel or its upstream signaling cascade. Previously, we demonstrated that the effects of mK_{Ca} channel activation by NS1619 on mitochondrial respiration were reduced in isolated cardiac mitochondria from aged rats. (10) Furthermore, there is evidence that aging is associated with decrease of K_{Ca} channel beta 1 subunit expression in the plasma membrane of coronary myocytes, (24) but it is completely unknown whether also mK_{Ca} channel expression is changed with increasing age. In the present study, we found that aging was without effect on mK_{Ca} channel beta 1 subunit expression, a finding that suggests that the aging-related loss of helium-induced cardioprotection is not caused by a decrease in mK_{Ca} channel density.

In summary, our results demonstrate that helium initiates preconditioning via activation of mK_{Ca} channels in the rat heart *in vivo*, that but that helium's protective potency is abolished in the senescent heart.

REFERENCES

1. **Abete P, Ferrara N, Cioppa A, Ferrara P, Bianco S, Calabrese C, Cacciatore F, Longobardi G and Rengo F**. Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiol* 27: 1777-1786, 1996.
2. **Bergmeyer HU**. *Methoden der enzymatischen Analyse*. Weinheim: Verlag Chemie, 1970.
3. **Cao CM, Xia Q, Gao Q, Chen M and Wong TM**. Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning. *J Pharmacol Exp Ther* 312: 644-650, 2005.
4. **Cribier A, Korsatz L, Koning R, Rath P, Gamra H, Stix G, Merchant S, Chan C and Letac B**. Improved myocardial ischemic response and enhanced collateral circulation with long repetitive coronary occlusion during angioplasty: a prospective study. *J Am Coll Cardiol* 20: 578-586, 1992.
5. **Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW, Jr., Herrmann HC and Laskey WK**. Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic, and metabolic features. *Circulation* 82: 2044-2051, 1990.
6. **Devlin W, Cragg D, Jacks M, Friedman H, O'Neill W and Grines C**. Comparison of outcome in patients with acute myocardial infarction aged > 75 years with that in younger patients. *Am J Cardiol* 75: 573-576, 1995.
7. **Haase KK, Schiele R, Wagner S, Fischer F, Burczyk U, Zahn R, Schuster S and Senges J**. In-hospital mortality of elderly patients with acute myocardial infarction: data from the

MITRA (Maximal Individual Therapy in Acute Myocardial Infarction) registry. *Clin Cardiol* 23: 831-836, 2000.

8. **Halestrap AP, Clarke SJ and Khaliulin I**. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 1767: 1007-1031, 2007.
9. **Heinen A, Camara AK, Aldakkak M, Rhodes SS, Riess ML and Stowe DF**. Mitochondrial Ca^{2+}-induced K^+ influx increases respiration and enhances ROS production while maintaining membrane potential. *Am J Physiol Cell Physiol* 292: C148-C156, 2007.
10. **Heinen A, Winning A, Schlack W, Hollmann MW, Preckel B, Frassdorf J and Weber NC**. The regulation of mitochondrial respiration by opening of mK_{Ca} channels is age-dependent. *Eur J Pharmacol* 578: 108-113, 2008.
11. **Juhaszova M, Rabuel C, Zorov DB, Lakatta EG and Sollott SJ**. Protection in the aged heart: preventing the heart-break of old age? *Cardiovasc Res* 66: 233-244, 2005.
12. **Lee TM, Su SF, Chou TF, Lee YT and Tsai CH**. Loss of preconditioning by attenuated activation of myocardial ATP-sensitive potassium channels in elderly patients undergoing coronary angioplasty. *Circulation* 105: 334-340, 2002.
13. **Liem DA, Manintveld OC, Schoonderwoerd K, McFalls EO, Heinen A, Verdouw PD, Sluiter W and Duncker DJ**. Ischemic preconditioning modulates mitochondrial respiration, irrespective of the employed signal transduction pathway. *Transl Res* 151: 17-26, 2008.
14. **Lim SY, Davidson SM, Hausenloy DJ and Yellon DM**. Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc Res* 75: 530-535, 2007.
15. **Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA and Downey JM**. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation* 84: 350-356, 1991.
16. **Liu Y and Downey JM**. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol* 263: H1107-H1112, 1992.
17. **Ljubkovic M, Mio Y, Marinovic J, Stadnicka A, Warltier DC, Bosnjak ZJ and Bienengraeber M**. Isoflurane preconditioning uncouples mitochondria and protects against hypoxia-reoxygenation. *Am J Physiol Cell Physiol* 292: C1583-C1590, 2007.
18. **Lowry OH, Rosebrough NJ, Farr AL and Randall RJ**. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
19. **Maggioni AP, Maseri A, Fresco C, Franzosi MG, Mauri F, Santoro E and Tognoni G**. Age-related increase in mortality among patients with first myocardial infarctions treated with thrombolysis. The Investigators of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI-2). *N Engl J Med* 329: 1442-1448, 1993.
20. **Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DM and Sack MN**. Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res* 89: 787-792, 2001.
21. **Moreau R, Heath SH, Doneanu CE, Harris RA and Hagen TM**. Age-related compensatory activation of pyruvate dehydrogenase complex in rat heart. *Biochem Biophys Res Commun* 325: 48-58, 2004.
22. **Murphy E and Steenbergen C**. Preconditioning: The mitochondrial connection. *Annu Rev Physiol* 69: 51-67, 2007.
23. **Murry CE, Jennings RB and Reimer KA**. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
24. **Nishimaru K, Eghbali M, Lu R, Marijic J, Stefani E and Toro L**. Functional and molecular evidence of MaxiK channel beta1 subunit decrease with coronary artery ageing in the rat. *J Physiol* 559: 849-862, 2004.
25. **O'Rourke B**. Evidence for mitochondrial K^+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.

26. **Obal D, Weber NC, Zacharowski K, Toma O, Dettwiler S, Wolter JI, Kratz M, Mullenheim J, Preckel B and Schlack W**. Role of protein kinase C-epsilon (PKCepsilon) in isoflurane-induced cardioprotection. *Br J Anaesth* 94: 166-173, 2005.
27. **Ohya S, Kuwata Y, Sakamoto K, Muraki K and Imaizumi Y**. Cardioprotective effects of estradiol include the activation of large-conductance Ca^{2+}-activated K^+ channels in cardiac mitochondria. *Am J Physiol Heart Circ Physiol* 289: H1635-H1642, 2005.
28. **Pagel PS, Krolikowski JG, Shim YH, Venkatapuram S, Kersten JR, Weihrauch D, Warltier DC and Pratt PF, Jr.** Noble gases without anesthetic properties protect myocardium against infarction by activating prosurvival signaling kinases and inhibiting mitochondrial permeability transition in vivo. *Anesth Analg* 105: 562-569, 2007.
29. **Sample J, Cleland JG and Seymour AM**. Metabolic remodeling in the aging heart. *J Mol Cell Cardiol* 40: 56-63, 2006.
30. **Shintani Y, Node K, Asanuma H, Sanada S, Takashima S, Asano Y, Liao Y, Fujita M, Hirata A, Shinozaki Y, Fukushima T, Nagamachi Y, Okuda H, Kim J, Tomoike H, Hori M and Kitakaze M**. Opening of Ca^{2+}-activated K^+ channels is involved in ischemic preconditioning in canine hearts. *J Mol Cell Cardiol* 37: 1213-1218, 2004.
31. **Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG and Jiang MT**. Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 290: H434-H440, 2006.
32. **Tani M, Suganuma Y, Hasegawa H, Shinmura K, Ebihara Y, Hayashi Y, Guo X and Takayama M**. Decrease in ischemic tolerance with aging in isolated perfused Fischer 344 rat hearts: relation to increases in intracellular Na^+ after ischemia. *J Mol Cell Cardiol* 29: 3081-3089, 1997.
33. **Toma O, Weber NC, Wolter JI, Obal D, Preckel B and Schlack W**. Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart in vivo. *Anesthesiology* 101: 1372-1380, 2004.
34. **Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A and O'Rourke B**. Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. *Science* 298: 1029-1033, 2002.

Chapter 6

Helium-induced early preconditioning is abolished in obese Zucker rats *in vivo*

[1] Ragnar Huhn, [1] André Heinen, Nina C. Weber, Raphaela Kerindongo, Markus W. Hollmann, Wolfgang Schlack, Benedikt Preckel

[1] These authors contributed equally to this work

Submitted for Publication

ABSTRACT

Ischemic preconditioning is abolished in the prediabetic Zucker obese rat. The noble gas helium has been shown to induce preconditioning by prevention of mitochondrial permeability transition pore (mPTP) opening. We investigated in Zucker rats if helium induces preconditioning and whether mitochondrial respiration or the Akt/ GSK-3beta signalling pathway as possible regulators of the mPTP are influenced. Chloralose-anesthetized Zucker rats were assigned to one of four groups. Zucker lean (ZL) and Zucker obese (ZO) control animals were not treated (ZL-/ZO-Con). Preconditioning groups (ZL-/ZO-He-PC) inhaled 70% helium for 3x5 minutes interspersed with 2x5 minutes and one final 10 minute washout period. Animals were exposed to 25 minutes regional myocardial ischemia followed by 120 minutes reperfusion. At the end of reperfusion, hearts were excised for infarct size measurement by TTC staining. In additional experiments, hearts were excised 5 min after the 3rd helium exposure for analysis of mitochondrial respiration and Western blot analysis of Akt and GSK-3beta phosphorylation. Helium-induced preconditioning reduced infarct size from 52±8% to 32±7% in ZL rats, but not in ZO rats (ZO-He-PC: 56±8% vs. ZO-Con: 54±9%). Mitochondrial respiration analysis showed that Helium causes mild uncoupling in ZL rats (2.27±0.07 vs. 2.51±0.10), but not in ZO rats (2.52±0.10 vs. 2.52±0.09). Helium pretreatment had neither in ZL nor in ZO rats an effect on Akt and GSK-3beta phosphorylation compared with respective controls. Helium-induced preconditioning is abolished in obese Zucker rats *in vivo* probably caused by a diminished effect of Helium on mitochondrial respiration.

INTRODUCTION

Diabetes mellitus is a known risk factor for the development of ischemic heart disease and myocardial infarction. (9) It was shown that acute myocardial infarction is consistently associated with an increased mortality in patients with type 2 diabetes. (7) Furthermore, diabetes mellitus is associated with a loss of the protective potency of cardioprotective strategies, e.g. preconditioning, both in humans and animals. (6; 7; 14) Katakam et al. (14) demonstrated that both ischemic preconditioning and pharmacological preconditioning by the mitochondrial ATP-activated potassium (mK_{ATP}) channel agonist diazoxide is abolished in Zucker obese rats, a widely used animal model of insulin resistance and type 2 diabetes.
Recently it was shown that exposure to the noble gas helium initiates a pronounced protection of the myocardium against ischemia reperfusion injury. (23) Helium is easy and safe to administer, and when compared to volatile anesthetics or xenon, the absence of anesthetic effects, as well as the lack of hemodynamic side effects would make helium an optimal agent for cardioprotection. (8; 16) These properties might offer the possibility to use helium in various groups of patients, e.g. during the perioperative period in patients at risk for cardiac events, as well as in non-surgical patients, e.g. in patients with instable angina or myocardial infarction. Helium is already safely used in the therapy of asthma and chronic obstructive pulmonary disease, as well as in young children with ventilation disorders. (8; 20)
It has also been shown that the cardioprotective effect of helium is mediated by activation of prosurvival signaling kinases and prevention of mitochondrial permeability transition pore (mPTP) opening. (23) Opening of the mPTP can be regulated by different mechanisms including alterations in mitochondrial bioenergetics or regulation of glycogen synthase kinase-3beta (GSK-3beta) activity, (10; 13) but the underlying mechanism by which helium confers cardioprotection by prevention of mPTP opening is unknown.
Based on these previous findings we aimed to investigate (1) if the noble gas helium initiates cardiac preconditioning in the Zucker obese rat *in vivo*, and (2) the underlying sub-cellular mechanism by which helium prevents mPTP opening, i.e. regulation of mitochondrial bioenergetics and/or inhibition of GSK-3beta dependent pathways.

MATERIALS AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication

No. 85-23, revised 1996), and approved by the Institutional Committee for Animal Care and Use (Academic Medical Center Amsterdam, The Netherlands). Animals had free access to food and water at all times before the start of the experiments.

Materials

Helium was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, the Netherlands). KCl was purchased from EMD Chemicals (Gibbstown, NJ, USA); all antibodies were purchased from Cell Signaling Technology Inc. (Danvers, USA) except the anti-α-tubulin and the anti-actin antibodies (Sigma, Saint Louis, USA). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands).

Surgical preparation

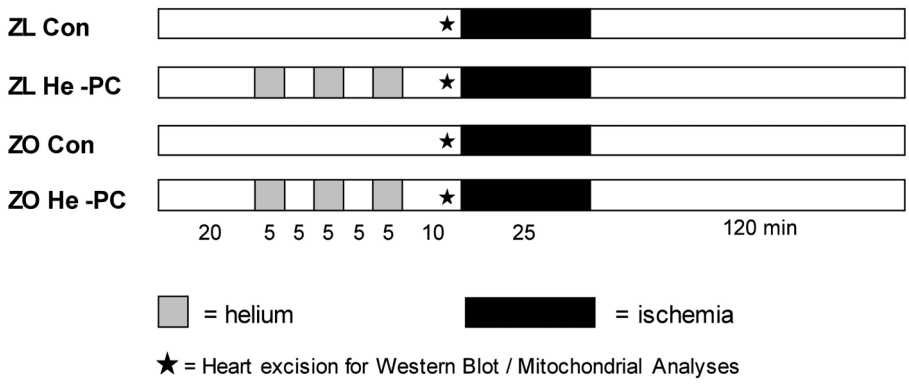
Male Zucker lean rats (253±28g) and male Zucker obese rats (322±31g) were anesthetized by intraperitoneal injection of S-ketamine (150 mg kg^{-1}) and Diazepam (1.5 mg kg^{-1}). Surgical preparation was performed as described previously. (25) Briefly, after tracheal intubation, the lungs were ventilated with oxygen-enriched (30% O_2) air and a positive end-expiratory pressure of 2-3 cm H_2O. Respiratory rate was adjusted to maintain PCO_2 within physiological limits. Body temperature was maintained at 38°C by the use of a heating pad. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. Anaesthesia was maintained by continuous α-chloralose infusion. A lateral left sided thoracotomy followed by pericardiotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery. All animals were left untreated for 20 minutes before the start of the respective experimental protocol. Aortic pressure was digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments Pty Ltd, Castle Hill, Australia).

Experimental protocol

Rats were divided into four groups (figure 1):

Animals for infarct size measurements underwent 25 min of coronary artery occlusion and 2 hours of reperfusion (I/R).

Figure 1: Experimental protocol



ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning

Zucker lean control group (ZL Con): After surgical preparation rats received 30% oxygen plus 70% nitrogen.
Zucker lean helium preconditioned group (ZL He-PC): Rats received helium 70% / 30% oxygen for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before I/R.
Zucker obese control group (ZO Con): After surgical preparation rats received 30% oxygen plus 70% nitrogen.
Zucker obese helium preconditioned group (ZO He-PC): Rats received helium 70% / 30% oxygen for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before I/R.

Infarct size measurement
After 120 minutes of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for perfusion with ice cold normal saline via the aortic root at a perfusion pressure of 80 cm H_2O in order to wash out intravascular blood. After 2 minutes of perfusion, the coronary artery was re-occluded and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 minutes. Intravascular Evans blue was then washed out by perfusion for 10 minutes with normal saline. This treatment identified the area at risk as unstained. The heart was then cut into

transverse slices, 2 mm thick. The slices were stained with 0.75% triphenyltetrazoliumchloride (TTC) solution for 10 minutes at 37°C, fixed in 4% formalin solution for 24 hours at room temperature. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight of each slide.

To investigate the effects of helium preconditioning on mitochondrial respiration and enzyme phosphorylation, e.g. GSK-3β (Ser9), and Akt (Thr308 and Ser473), additional experiments (n = 8 for each group) were conducted using the same preconditioning protocol except that the hearts were excised 5 min after the third helium administration (see figure 1).

Mitochondrial isolation

Heart mitochondria were isolated by differential centrifugation as described previously. (12) Briefly, atria were removed and ventricles were placed in isolation buffer [200 mmol/L mannitol, 50 mmol/L sucrose, 5 mmol/L KH_2PO_4, 5 mmol/L 3-(n-morpholino) propanesulfonic acid (MOPS), 1 mmol/L Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1% bovine serum albumin (BSA), pH 7.15 adjusted with KOH], and minced into 1 mm^3 pieces. The suspension was homogenized for 15 sec in 2.5 ml isolation buffer containing 5 U/ml protease, and for another 15 sec after addition of 17 ml isolation buffer. The suspension was centrifuged at 3220*g* for 10 min, the supernatant was removed, and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 3220*g* for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. Protein content was determined by the Bradford method. (1) All isolation procedures were conducted at 4°C.

Mitochondrial respiration

Oxygen consumption was measured polarographically at 37°C using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.3 mg protein/ml) were suspended in respiration buffer containing 130 mmol/L KCl, 5 mmol/L K_2HPO_4, 20 mmol/L MOPS, 2.5 mmol/L EGTA, 1 µmol/L $Na_4P_2O_7$, 0.1% BSA, pH 7.15 adjusted with KOH. Mitochondrial respiration was initiated by administration of 10 mmol/L complex II substrate succinate (+10 µmol/L complex I blocker rotenone) after 60 sec. State 3 respiration was initiated after 120 sec by addition of 200 µmol/L adenosine-diphosphate (ADP). Respiration rates were recorded under state 3 conditions and after

complete phosphorylation of ADP to adenosine-triphosphate (ATP) (State 4). The respiratory control index (RCI, state 3/state 4) and the P/O ratio (phosphate incorporated into ATP to oxygen consumed) were calculated as parameter of mitochondrial coupling between respiration and oxidative phosphorylation, and mitochondrial efficiency, respectively. From each heart, respiration measurements were repeated in 3 mitochondrial samples and the average was taken (and counted as n = 1). Respiration rates are expressed as absolute rates in nmol O_2/mg/min.

Separation of cytosolic fraction

For cellular fractionation and subsequent Western blot assay, tissue specimens were prepared for protein analysis of GSK-3β (Ser9) and Akt (Thr308 and Ser473), respectively. The excised hearts were frozen in liquid nitrogen. Subsequently, a cellular fractionation was performed as described previously. (27) The frozen tissue was pulverized and dissolved in lysis buffer containing: Tris base, EGTA, NaF and Na_3VO_4 (as phosphatase inhibitors), a freshly added protease inhibitor mix (aprotinin, leupeptin and pepstatin) and DTT. The solution was vigorously homogenized on ice (Homogenisator, IKA) and then centrifuged at 1000 *g*, 4°C, for 10 min. The supernatant, containing the cytosolic fraction, was centrifuged again at 16000 *g*, 4°C, for 15 min to clean up this fraction for further Western blot assay.

Western blot analysis

After protein concentration was determined by the Lowry method (18) equal amounts of protein were prepared and loaded on a 10% SDS-PAGE gel. The proteins were separated by electrophoresis (100 V, 85 min) and then transferred to a PVDF membrane by tank blotting (100V, 1h). To prevent unspecific antibody binding the membrane was subsequently blocked with 5% skimmed milk solution in Tris buffered saline containing Tween (TBS-T) for 2 hours. Then, the membrane was incubated over night at 4°C with the respective primary antibody GSK-3beta (1:10000), Akt(Thr308)(1:1000), or Akt(Ser473)(1:1000). After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands were visualized by chemiluminescence detected on X-ray film (Hyperfilm ECL, Amersham) using the enhanced chemiluminescence system Santa Cruz. The blots were quantified using a Kodak Image station® (Eastman Kodak Comp., Rochester, NY) and the results are presented as ratio of phosphorylated protein to total

protein. Equal loading of protein on the gel was additionally proved by detection of α-tubulin or actin, respectively, and Coomassie blue staining of the gels.

Statistical Analysis

Data are expressed as mean ± SD. Heart rate (HR) and mean aortic pressure (AOPmean) were measured during baseline, coronary artery occlusion, and reperfusion period. Inter-group differences of hemodynamic data were analyzed (SPSS Science Software, version 12.0.1) by performing an one-way ANOVA followed by Tukey's post-hoc test. Time effects (changes from baseline value) during the experiments were analyzed by using an one-way ANOVA followed by Dunnett's post-hoc test. Infarct sizes were analyzed by an one-way ANOVA followed by Tukey's post-hoc test. Changes within and between groups were considered statistically significant if $P<0.05$.

RESULTS

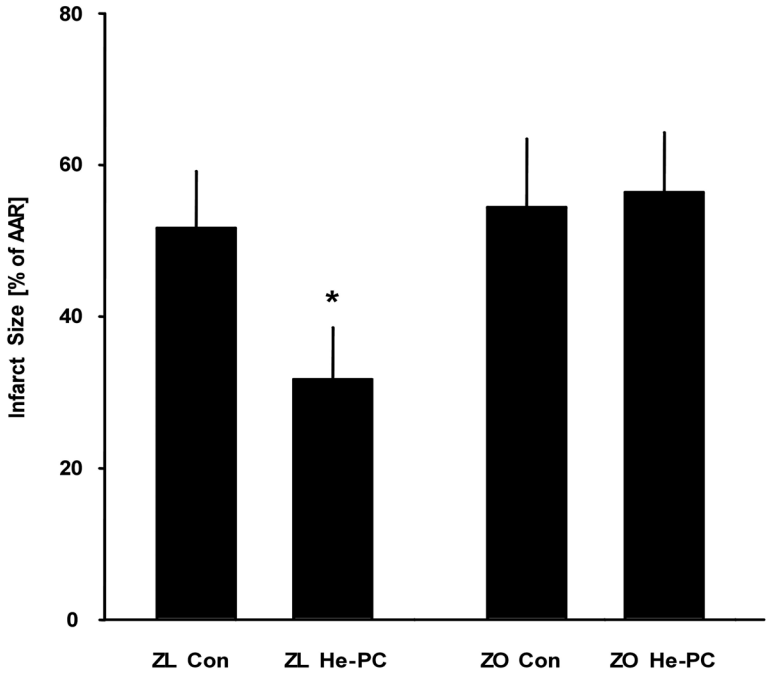
Infarct size measurement

Helium preconditioning reduced infarct size in ZL rats from 52 ± 8 % in controls (n = 8) to 32 ± 7 % (n = 8, $p<0.05$, Fig. 2). In ZO control rats (n = 8), infarct size was similar to ZL controls (54 ± 9 %, n = 8, n.s. vs. ZL Con). In contrast to the protection seen in ZL rats, in ZO rats did helium not reduce infarct size (56 ± 8 %, n = 8, n.s.; Fig. 2).

Hemodynamic variables

Hemodynamic variables are summarized in table 1. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischemia and reperfusion. At the end of the experiments, mean aortic pressure was significantly decreased compared with baseline in all groups with the exception of the ZO control group.

Figure 2: Infarct size



Histogram shows the infarct sizes as percent of area at risk (AAR). ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning. Data are presented as mean ± SD, *p < 0.05 vs. ZL Con

Weights and blood glucose levels

The body weights of ZO rats (ZO Con: 315 ± 33, and ZO He-PC: 330 ± 29) were significant higher than in ZL rats (ZL Con: 244 ± 27, and ZL He-PC: 262 ± 27) (table 2). Blood glucose levels were not different between groups.

Table 1: Hemodynamic variables

	Baseline	Washout 3	Ischemia	Reperfusion		
			15 min	30 min	60 min	120 min
Heart Rate (BPM)						
ZL Con	419 ± 26	428 ± 27	435 ± 23	378 ± 48	375 ± 58	399 ± 60
ZL He-PC	417 ± 34	420 ± 30	423 ± 22	387 ± 32	364 ± 24*	351 ± 39*
ZO Con	414 ± 28	397 ± 27	406 ± 44	381 ± 45	360 ± 47	349 ± 53
ZO He-PC	416 ± 38	410 ± 23	415 ± 21	398 ± 32	387 ± 31	372 ± 45
Mean AOP (mmHg)						
ZL Con	116 ± 18	105 ± 25	96 ± 21	80 ± 17*	81 ± 27*	69 ± 17*
ZL He-PC	120 ± 20	103 ± 13	90 ± 22	74 ± 24*	62 ± 22*	67 ± 24*
ZO Con	111 ± 33	106 ± 37	100 ± 41	81 ± 27	73 ± 26	66 ± 24
ZO He-PC	120 ± 11	113 ± 13	107 ± 20	91 ± 28	81 ± 31*	60 ± 24*

Data are Mean ± SD. ZL = Zucker lean; ZO = Zucker obese; Con = control group; He-PC = Helium preconditioning; AOP = aortic pressure. *P<0.05 vs. baseline.

Mitochondrial respiration

Mitochondrial respiration results are summarized in figure 3. There was no significant difference in the RCI between ZL (n = 8) and ZO (n = 7) control rats (2.51 ± 0.10 vs. 2.52 ± 0.09, n.s.). Helium preconditioning reduced the RCI in ZL rats (2.27 ± 0.07; n = 8; $p<0.05$ vs. ZL Con), but had no effect on the RCI in ZO rats (2.52 ± 0.10; n = 8; n.s. vs. ZO Con). The reduction in the RCI in ZL He-PC was caused by an increase in state 4 respiration (155 ± 11 nmol O_2/mg/min vs. 139 ± 10 nmol O_2/mg/min, $p<0.05$); state 3 respiration was not affected by helium preconditioning in both ZL and ZO rats.

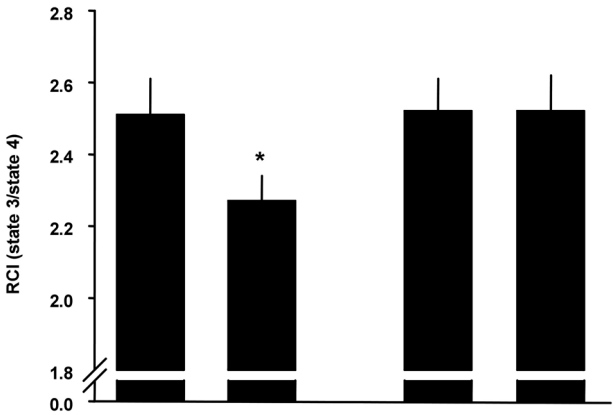
There was no difference between all groups in the efficiency of oxidative phosphorylation as demonstrated by no changes in the P/O ratio.

Table 1: Blood glucose levels and weights

Group	Blood sugar (mmol l^{-1})	Body weight (g)	Heart dry weight (g)
ZL Con	6.7 ± 0.3	244 ± 27	0.18 ± 0.02
ZL He-PC	6.3 ± 1.7	262 ± 27	0.17 ± 0.02
ZO Con	6.9 ± 1.1	315 ± 33*	0.18 ± 0.02
ZO He-PC	7.8 ± 2.1	330 ± 29*	0.19 ± 0.02

ZL = Zucker lean; ZO = Zucker obese; Con = control; He-PC = helium preconditioning. Data are Mean ± SD.*P<0.05 vs. ZL Con

Figure 3: Mitochondrial respiration



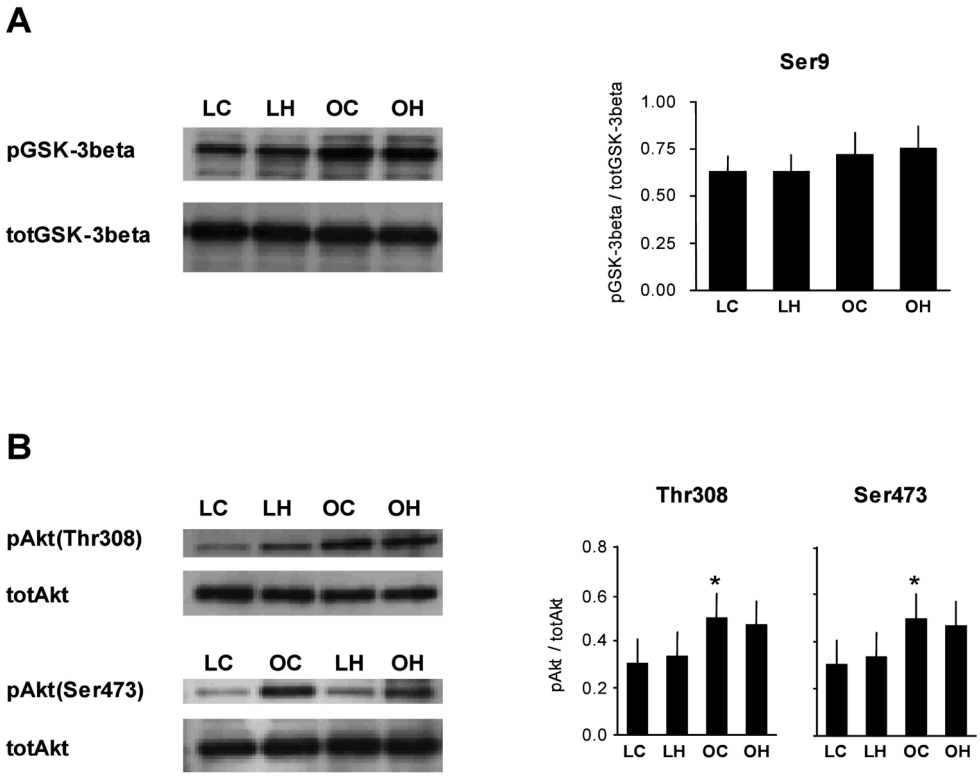
	ZL Con	ZL He-PC	ZO Con	ZO He-PC
state 3 (nmol O_2/mg/min)	349 ±18	349 ±21	344 ±22	348 ±15
state 4 (nmol O_2/mg/min)	139 ±10	155 ±11 *	136 ±12	139 ±5
P/O ratio	1.54 ±0.07	1.53 ±0.09	1.56 ±0.04	1.55 ±0.07

Summarized data for the effects of helium preconditioning on mitochondrial respiration. ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning. RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into ATP and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation. Data are presented as mean ± SD, *p < 0.05 vs. ZL Con.

Regulation of GSK-3beta and Akt phosphorylation during helium preconditioning

Figure 4 A shows that there was no difference in the phosphorylation state of GSK-3beta (Ser9) between ZL control and ZO control rat hearts (0.63 ± 0.08 vs. 0.72 ± 0.12, n.s.).

Figure 4: Western blot analysis



Effects of Helium-induced preconditioning on GSK-3beta (Ser9) (panel A) and Akt (Thr308 and Ser473) phosphorylation. Representative Western blots (left) and summarized data presenting ratio of phosphorylated enzyme to total enzyme (right) are shown. LC = Zucker lean control, OC = Zucker obese control, LH = Zucker lean helium preconditioning, OH = Zucker obese helium preconditioning. Data are presented as mean ± SD, *p < 0.05 vs. LC.

Pretreatment with helium had neither an effect on the phosphorylation state of GSK-3beta (Ser9) in ZL rats (0.63 ± 0.09 vs. 0.63 ± 0.08, n.s.) nor in ZO rats (0.75 ± 0.12 vs. 0.72 ± 0.12, n.s.).

Figure 4 B shows that there was a significant increase in the phosphorylation state of both Akt(Thr308) and Akt(Ser473) between ZL control and ZO control rat hearts (Akt(Thr308): 0.43 ± 0.19 vs. 0.64 ± 0.11, $p<0.05$; Akt(Ser473): 0.30 ± 0.10 vs. 0.50 ± 0.11, $p<0.05$). Pretreatment with helium had neither an effect on the phosphorylation state of Akt(Thr308) nor Akt(Ser473) in both ZL rats (Akt(Thr308): 0.44 ± 0.09 vs. 0.43 ± 0.19, n.s.; Akt(Ser473): 0.33 ± 0.10 vs. 0.30 ± 0.10, n.s., respectively) and ZO rats(Akt(Thr308): 0.59 ± 0.07 vs. 0.64 ± 0.11, n.s.; Akt(Ser473): 0.47 ± 0.10 vs. 0.50 ± 0.11, n.s., respectively) .

DISCUSSION

The main findings of our study are that the cardioprotective effect of helium-induced preconditioning *a)* is abolished in the prediabetic rat heart, and *b)* is mediated rather by regulation of mitochondrial respiration, i.e. mild mitochondrial uncoupling, than by activation of prosurvival signaling kinases.

It is well known that besides brief periods of ischemia, also pharmacological interventions, e.g. administration of adenosin, potassium channel agonists, or volatile anesthetics can initiate cardiac preconditioning to enhance the resistance of the myocardium against ischemia and reperfusion injury. (2-4; 15; 19) Very recently it was demonstrated that pharmacological preconditioning can also be initiated by the noble gas helium, which is a non-anesthetic gas without significant hemodynamic side effects. (23) Therefore, helium would be an ideal agent for cardioprotection in patients with cardiovascular disease not only in the perioperative setting like during cardiac surgery, but also for interventional procedures like during percutaneous coronary interventions.

There is evidence that the protective potency of both ischemic and pharmacological preconditioning is diminished in the diabetic heart. (14; 17; 26) In the present study, we show that besides the rabbit heart (23) also the rat heart can be preconditioned by helium. Furthermore, we demonstrate that the cardioprotective effect of helium is abolished in the prediabetic rat heart. Kristiansen et al. (17) demonstrated in obese Zucker diabetic fatty and lean Goto-Kakizaki rats, two widely used rat models of type 2 diabetes, that ischemic preconditioning does not reduce infarct size. In contrast, Tsang et al. (26) showed that preconditioning can be induced in hearts from Goto-Kakizaki rats, but the threshold that is required to achieve preconditioning is elevated in diabetic compared with non diabetic hearts. The experimental diabetes models that were used in both studies were characterized by a significant hyperglycemia. Very recently, Katakam et al. (14) demonstrated that both, ischemic and pharmacological preconditioning induced by the mK_{ATP} channel agonist

diazoxide, are abolished in Zucker obese rats. The experiments were conducted in 10-12 weeks old rats. At this age, ZO rats are hyperinsulinemic and normoglycemic, (22) representing a prediabetic state of type 2 diabetes. In the present study, we used 10-12 weeks old ZO rats that were normoglycemic, and our results are in line with the finding by Katakam et al. (14) showing that cardioprotection is abolished in the prediabetic heart.

The mechanism by which helium-induced preconditioning is blocked in the prediabetic heart is unknown. Hassouna et al. (11) investigated the ability to induce ischemic and pharmacological preconditioning of the diabetic human myocardium. Their results showed that ischemic and pharmacological preconditioning failed to protect the diabetic myocardium, possibly caused by dysfunctional potassium channels in the inner mitochondrial membrane. Alterations in mitochondrial function caused by potassium channel activation have been proposed to protect the myocardium by reducing mPTP opening. (10) Pagel et al. (23) demonstrated that helium-induced preconditioning is abrogated by administration of the mPTP opener atractyloside showing a central role of mPTP in helium-induced preconditioning. The mPTP is a multiprotein complex formed at the contact sites between the inner and the outer mitochondrial membrane by several proteins including the adenine nucleotide translocase, the voltage-dependent anion channel, cyclophilin D, and hexokinase. (5) In the normal myocardium, the inner mitochondrial membrane is relatively impermeable, as it must be to maintain the proton gradient (the driving force for oxidative phosphorylation) established by the mitochondrial respiratory chain. Opening of the mPTP leads to a sudden increase in permeability of the inner mitochondrial membrane, loss of mitochondrial membrane potential, excessive swelling of the mitochondrial matrix with the consequence of the outer mitochondrial membrane rupture and release of proteins into the cytosol [including cytochrome C]. (24) It has been suggested that preconditioning prevents mPTP opening by regulation of prosurvival signaling kinases including Akt and GSK-3beta, and/or by regulation of mitochondrial bioenergetics, i.e. mild uncoupling of mitochondrial respiration. (10; 21) Our results demonstrate that in Zucker lean rats helium causes a reduction of the respiratory control index while it did not cause phosphorylation of both GSK-3beta and Akt. From these data we conclude that the prevention of mPTP opening in helium-induced preconditioning is mediated by mild uncoupling of mitochondrial respiration. This conclusion is further supported by the finding that helium did not induce preconditioning in Zucker obese rats, which is in line with our findings that helium has no effect on mitochondrial respiration in these prediabetic rats.

The role of prosurvival signaling kinases in helium-induced preconditioning remains unclear. Pagel et al. demonstrated in the rabbit heart that the protective effect of helium was blocked by pharmacological inhibition of phosphatidylinositol-3-kinase, extracellular signal-regulated kinase, and 70-kDa ribosomal protein s6 kinase. In the current study, we did not detect an effect of helium on Akt and GSK-3beta phosphorylation. There are several possible explanations including species differences and lack of drug specifity that could cause this discrepancy. Interestingly, the importance of Akt phosphorylation in the signal transduction of ischemic preconditioning was demonstrated by Tsang et al. (26) In their study, preconditioning caused an increased Akt phosphorylation 5 min after the last preconditioning cycle, i.e. the same timing of tissue sampling as we used in the present study.

Taken together, the present study demonstrates that the noble gas helium can induce preconditioning in the rat heart *in vivo*. The protective effect could be explained by mild mitochondrial uncoupling, an alteration that is capable to prevent mPTP opening. Furthermore, the protective potency of helium-induced preconditioning is completely abrogated in the Zucker obese rat, a widely used animal model for prediabetic conditions of state 2 diabetes. Whether this cardioprotection can be reestablished in the prediabetic heart by further pharmacological intervention needs further investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Gezina T.M.L. Oei for her support with this study.

REFERENCES

1. **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
2. **Cason BA, Gamperl AK, Slocum RE and Hickey RF**. Anesthetic-induced preconditioning: previous administration of isoflurane decreases myocardial infarct size in rabbits. *Anesthesiology* 87: 1182-1190, 1997.
3. **Cohen MV, Yang XM, Liu GS, Heusch G and Downey JM**. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K_{ATP} channels. *Circ Res* 89: 273-278, 2001.
4. **Cope DK, Impastato WK, Cohen MV and Downey JM**. Volatile anesthetics protect the ischemic rabbit myocardium from infarction. *Anesthesiology* 86: 699-709, 1997.
5. **Crompton M**. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341 (Pt 2): 233-249, 1999.

6. **del Valle HF, Lascano EC, Negroni JA and Crottogini AJ**. Absence of ischemic preconditioning protection in diabetic sheep hearts: role of sarcolemmal K_{ATP} channel dysfunction. *Mol Cell Biochem* 249: 21-30, 2003.
7. **Donnan PT, Boyle DI, Broomhall J, Hunter K, MacDonald TM, Newton RW and Morris AD**. Prognosis following first acute myocardial infarction in Type 2 diabetes: a comparative population study. *Diabet Med* 19: 448-455, 2002.
8. **Gainnier M and Forel JM**. Clinical review: use of helium-oxygen in critically ill patients. *Crit Care* 10: 241, 2006.
9. **Haffner SM, Lehto S, Ronnemaa T, Pyorala K and Laakso M**. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 339: 229-234, 1998.
10. **Halestrap AP, Clarke SJ and Khaliulin I**. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 1767: 1007-1031, 2007.
11. **Hassouna A, Loubani M, Matata BM, Fowler A, Standen NB and Galinanes M**. Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium. *Cardiovasc Res* 69: 450-458, 2006.
12. **Heinen A, Winning A, Schlack W, Hollmann MW, Preckel B, Frassdorf J and Weber NC**. The regulation of mitochondrial respiration by opening of mK_{Ca} channels is age-dependent. *Eur J Pharmacol* 578: 108-113, 2008.
13. **Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN and Sollott SJ**. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 113: 1535-1549, 2004.
14. **Katakam PV, Jordan JE, Snipes JA, Tulbert CD, Miller AW and Busija DW**. Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. *Am J Physiol Regul Integr Comp Physiol* 292: R920-R926, 2007.
15. **Kersten JR, Schmeling TJ, Pagel PS, Gross GJ and Warltier DC**. Isoflurane mimics ischemic preconditioning via activation of K(ATP) channels: reduction of myocardial infarct size with an acute memory phase. *Anesthesiology* 87: 361-370, 1997.
16. **Koblin DD, Fang Z, Eger EI, Laster MJ, Gong D, Ionescu P, Halsey MJ and Trudell JR**. Minimum alveolar concentrations of noble gases, nitrogen, and sulfur hexafluoride in rats: helium and neon as nonimmobilizers (nonanesthetics). *Anesth Analg* 87: 419-424, 1998.
17. **Kristiansen SB, Lofgren B, Stottrup NB, Khatir D, Nielsen-Kudsk JE, Nielsen TT, Botker HE and Flyvbjerg A**. Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. *Diabetologia* 47: 1716-1721, 2004.
18. **Lowry OH, Rosebrough NJ, Farr AL and Randall RJ**. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
19. **Murry CE, Jennings RB and Reimer KA**. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
20. **Myers TR**. Use of heliox in children. *Respir Care* 51: 619-631, 2006.
21. **Nishihara M, Miura T, Miki T, Tanno M, Yano T, Naitoh K, Ohori K, Hotta H, Terashima Y and Shimamoto K**. Modulation of the mitochondrial permeability transition pore complex in GSK-3beta-mediated myocardial protection. *J Mol Cell Cardiol* 43: 564-570, 2007.
22. **Oltman CL, Richou LL, Davidson EP, Coppey LJ, Lund DD and Yorek MA**. Progression of coronary and mesenteric vascular dysfunction in Zucker obese and Zucker diabetic fatty rats. *Am J Physiol Heart Circ Physiol* 291: H1780-H1787, 2006.
23. **Pagel PS, Krolikowski JG, Shim YH, Venkatapuram S, Kersten JR, Weihrauch D, Warltier DC and Pratt PF, Jr.** Noble gases without anesthetic properties protect

myocardium against infarction by activating prosurvival signaling kinases and inhibiting mitochondrial permeability transition in vivo. *Anesth Analg* 105: 562-569, 2007.

24. **Scarlett JL and Murphy MP**. Release of apoptogenic proteins from the mitochondrial intermembrane space during the mitochondrial permeability transition. *FEBS Lett* 418: 282-286, 1997.
25. **Toma O, Weber NC, Wolter JI, Obal D, Preckel B and Schlack W**. Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart in vivo. *Anesthesiology* 101: 1372-1380, 2004.
26. **Tsang A, Hausenloy DJ, Mocanu MM, Carr RD and Yellon DM**. Preconditioning the diabetic heart: the importance of Akt phosphorylation. *Diabetes* 54: 2360-2364, 2005.
27. **Weber NC, Toma O, Damla H, Wolter JI, Schlack W and Preckel B**. Upstream signaling of protein kinase C-epsilon in xenon-induced pharmacological preconditioning. Implication of mitochondrial adenosine triphosphate dependent potassium channels and phosphatidylinositol-dependent kinase-1. *Eur J Pharmacol* 539: 1-9, 2006.

Chapter 7

Helium-induced late preconditioning in the rat heart *in vivo*

[1] Ragnar Huhn, [1] André Heinen, Nina C. Weber, Stefanie Hieber, Markus W. Hollmann, Wolfgang Schlack, Benedikt Preckel

[1] These authors contributed equally to this work

Submitted for Publication

ABSTRACT

The noble gas helium induced early myocardial preconditioning. We investigated if helium induces also late preconditioning, and if so, whether the cardioprotective effect is concentration-dependent. In a second part we studied whether repeated administration of helium on subsequent days will result in an increased cardioprotection. The study consisted of two parts. Chloralose anesthetized Wistar rats were subjected to 25 min of coronary artery occlusion followed by 120 min of reperfusion (I/R). In part 1, late preconditioning was achieved by administration of 70%, 50% and 30% helium for 15 minutes 24 hours before I/R. Based on findings of part 1, in additional experiments 30% helium was administered subsequently on three and two days before I/R. At the end of the experiments hearts were excised for infarct size measurements. Additional experiments were performed for mitochondrial respiration and Western blot analysis of cyclooxygenase-2 (COX-2). In the control group, infarct size was 55±8 % of the area at risk. All three helium concentrations reduced infarct size (He-LPC 70: 37±13%, He-LPC 50: 34±16%, He-LPC 30: 40±9%; each $P<0.05$ vs. Con). Repeated administration of helium more than one time did not further enhance cardioprotection (He-LPC 3x30: 39±9 %, He-LPC 2x30: 38±10%; $P>0.05$ vs. He-LPC 1x30: 37±11%). There were no differences in COX-2 expression and mitochondrial function after helium preconditioning. We show for the first time that helium induces late preconditioning and that cardioprotection is already maximal with administration of one cycle of 30% helium.

INTRODUCTION

Cardioprotection by preconditioning consists of two phases, an early phase (EPC), disappearing between 2 and 3 hours after the preconditioning stimulus, and a late phase (LPC), reappearing 24 hours after the initial stimulus and lasting for 2-3 days. (2) Both, EPC and LPC can be induced by various stimuli, e.g. brief ischemia or pharmacological agents. (3; 9; 17) LPC can also be mimicked by administration of volatile anesthetics. (7; 28) A recent study showed that the noble gas helium (He) induces early preconditioning of the heart *in vivo*. (22) It is unknown whether helium can also induce late preconditioning (He-LPC), and whether a possible cardioprotective effect is concentration-dependent. We hypothesized that helium induces late preconditioning and determined in the first part of the study whether concentrations lower than 70% can induce late preconditioning.

It is known that the effect of preconditioning depends on the severity of the stimulus. (1) Therefore we hypothesized that a preconditioning stimulus that is given on several subsequent days can enhance cardioprotection by LPC and investigated whether repeated administration of helium on various days within the time window of late preconditioning will lead to further myocardial protection.

It has been demonstrated that cyclooxygenase-2 (COX-2) plays a central role as mediator of both, ischemic and pharmacological induced LPC. (4; 14) Furthermore, Pagel et al. showed that prevention of mitochondrial permeability transition pore (mPTP) opening is involved in He-EPC, but its involvement in LPC has yet not been determined. (22) A possible mechanism by which mPTP opening is prevented is regulation of mitochondrial respiration, e.g. mild mitochondrial uncoupling. Therefore, we hypothesized that helium-induced late preconditioning is mediated by COX-2 and/or mitochondrial uncoupling and investigated these effects at different time points after helium administration.

MATERIALS AND METHODS

The study was performed in accordance with the requirements of the Animal Ethics Committee of the University of Amsterdam and was in line with European Union directives on the care and use of experimental animals.

Helium-induced late preconditioning

Materials

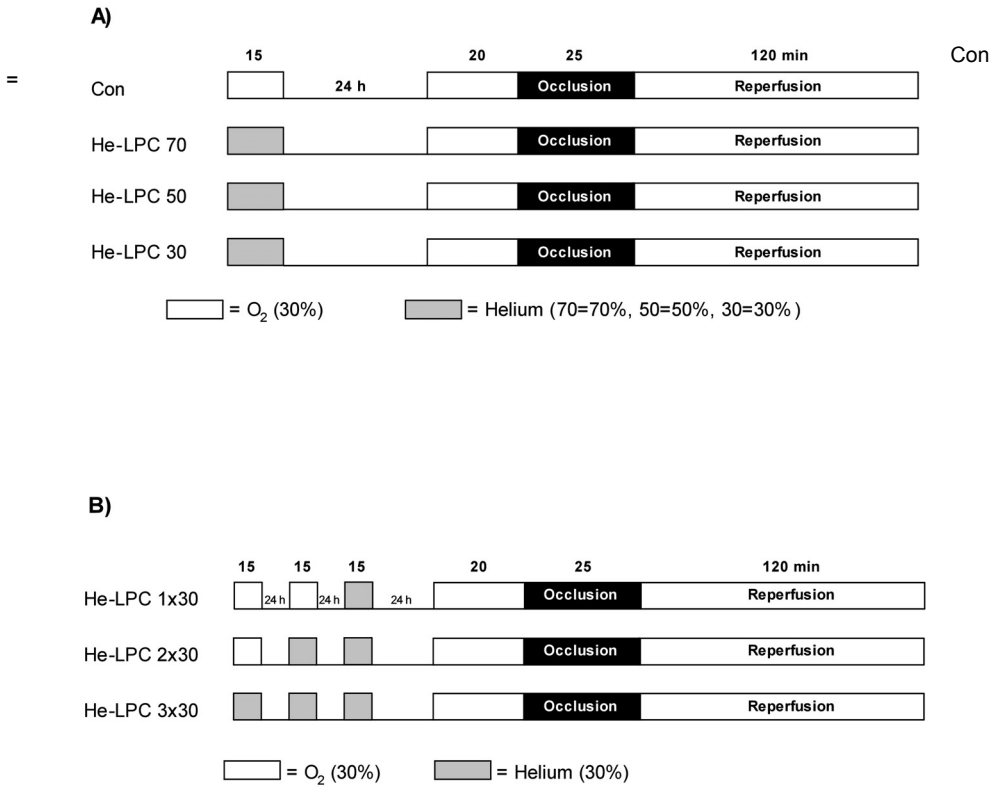
Helium was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, the Netherlands). Mouse Anti-Cyclooxygenase-2 monoclonal antibody was purchased from bio connect (bio connect, Huissen, The Netherlands). KCl was purchased from EMD Chemicals (Gibbstown, NJ); all other chemicals were purchased from Sigma Chemical Co. (Taufkirchen, Germany).

Experimental protocol for infarct size determination:

Animals had free access to food and water at all times before the start of the experiments. Male Wistar rats (328 ± 19 g) were anesthetized by intraperitoneal S-ketamine injection (150 mg/kg). S-ketamine does not interfere with cardioprotection in animals *in vivo*. (19)
On the day of the final experiment all animals underwent 25 min of coronary artery occlusion and 2 hours of reperfusion (I/R). In part one of the study, rats were divided into four groups (Fig. 1A):

Control group (Con): 24 hours before I/R rats received 30% oxygen for 15 minutes. On the day of the experiment rats received 30% oxygen plus 70% nitrogen after surgical preparation.
70% Helium late preconditioned group (He-LPC 70): 24 hours before I/R, rats received 70% Helium and 30% oxygen for 15 minutes. On the day of the experiment rats received 30% oxygen plus 70% nitrogen after surgical preparation.
50% Helium late preconditioned group (He-LPC 50): 24 hours before I/R, rats received 50% Helium, 30% oxygen and 20% nitrogen for 15 minutes. On the day of the experiment rats received 30% oxygen plus 70% nitrogen after surgical preparation.
30% Helium late preconditioned group (He-LPC 30): 24 hours before I/R, rats received 30% Helium, 30% oxygen and 40% nitrogen for 15 minutes. On the day of the experiment rats received 30% oxygen plus 70% nitrogen after surgical preparation.
In part two of the study rats were divided into three groups (Fig. 1B):
Helium late preconditioned group (He-LPC 3x30, He-LPC 2x30 or He-LPC 1x30): rats received 30% Helium, 30% oxygen and 40% nitrogen for 15 minutes on three, two or one day(s), respectively. On the day of the experiment rats received 30% oxygen plus 70% nitrogen after surgical preparation.

Figure 1: Experimental protocol



Control, He = Helium, LPC = late preconditioning.

For mitochondrial respiration and Western Blot analysis, additional experiments were performed to investigate time effects of helium. Hearts were excised 6, 10, or 24 hours after helium (30% Helium, 30% oxygen and 40% nitrogen) inhalation for 15 minutes (He-LPC 6h, He-LPC 10h, He-LPC 24h, respectively). Hearts from control animals (Con) were excised immediately after inhalation of 30% oxygen and 70% nitrogen for 15 minutes. Rats underwent a sham operation and recovered for 20 minutes before hearts were excised in order to follow the same surgical protocol as in the infarct size experiments.

Surgical preparation and infarct size measurement:
Surgical preparation was performed as described previously. (21; 29) In brief, male Wistar rats (328 ± 19 g) were anesthetized by intraperitoneal S-ketamine injection (150 mg/kg). After tracheal intubation, the lungs were ventilated with oxygen-enriched air and a positive

end-expiratory pressure of 2-3 cm H_2O. Respiratory rate was adjusted to maintain PCO_2 within physiological limits. Body temperature was maintained at 38°C by the use of a heating pad. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. Anesthesia was maintained by continuous α-chloralose infusion. A lateral left sided thoracotomy followed by pericardiotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery. All animals were left untreated for 20 minutes before the start of the respective experimental protocol. Arterial blood gases were analyzed at baseline and PCO_2 and PO_2 were kept within physiological ranges by adjusting ventilation. During the experiment the oxygen concentration was measured in the expiratory gas (Datex Capnomac Ultima, Division of Instrumentarium Corp., Helsinki, Finland). Aortic pressure and electrocardiographic signals were digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments).

After 120 minutes of reperfusion, the heart was excised and infarct size was determined as previously described.(21) The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL).

Mitochondrial isolation

Heart mitochondria were isolated by differential centrifugation as described previously. (12) Briefly, atria were removed and ventricles were placed in isolation buffer and minced into 1 mm^3 pieces. The suspension was homogenized for 15 sec in 2.5 ml isolation buffer containing 5 U/ml protease (from Bacillus licheniformis, Enzyme Commission Number 3.4.21.14), and for another 15 sec after addition of 17 ml isolation buffer. The suspension was centrifuged at 3220*g* for 10 min, the supernatant was removed, and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 3220*g* for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. Protein content was determined by the Bradford method. (5) All isolation procedures were conducted at 4°C.

Mitochondrial respiration

Oxygen consumption was measured polarographically at 37°C using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.3 mg

protein/ml) were suspended in respiration buffer containing 130 mmol/L KCl, 5 mmol/L K_2HPO_4, 20 mmol/L 3-(N-Morpholino)propanesulfonic acid (MOPS), 2.5 mmol/L Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 µmol/L $Na_4P_2O_7$, 0.1% bovine serum albumine (BSA), pH 7.15 adjusted with KOH. Mitochondrial respiration was initiated by administration of 10 mmol/L complex II substrate succinate (+10 µmol/L complex I blocker rotenone) after 60 sec. State 3 respiration was initiated after 120 sec by addition of 200 µmol/L adenosine-diphosphate (ADP). Respiration rates were recorded under state 3 conditions and after complete phosphorylation of ADP to adenosine-triphosphate (ATP) (State 4). The respiratory control index (RCI, state 3/state 4) and the P/O ratio (phosphate incorporated into ATP to oxygen consumed) were calculated as parameter of mitochondrial coupling between respiration and oxidative phosphorylation, and mitochondrial efficiency, respectively. From each heart, respiration measurements were repeated in 3 mitochondrial samples and the average was taken (and counted as n=1). Respiration rates are expressed as absolute rates in nmol O_2/mg/min.

Separation of membrane and cytosolic fraction

For cellular fractionation and subsequent Western blot assay, tissue specimens were prepared for protein analysis and distribution (membrane-, cytosolic-fraction) of phosphorylated COX-2 within the myocytes. The excised hearts were frozen in liquid nitrogen. Subsequently, a cellular fractionation was performed that was adapted from the literature. (6; 13; 16) The frozen tissue was pulverized and dissolved in lysis buffer containing: Tris base, EGTA, NaF and Na_3VO_4 (as phosphatase inhibitors), a freshly added protease inhibitor mix (aprotinin, leupeptin and pepstatin) and DTT. The solution was vigorously homogenized on ice (Homogenisator, IKA, Staufen, Germany) and then centrifuged at 1000 *g*, 4°C, for 10 min. The supernatant, containing the cytosolic fraction, was centrifuged again at 16000 *g*, 4°C, for 15 min to clean up this fraction. The remaining pellet was resuspended in lysis buffer containing 1% Triton X 100, incubated for 60 min on ice and vortexed. The solution was centrifuged at 16000 *g*, 4°C, for 15 min. The supernatant containing the membrane fraction was stored at -80°C until use for further Western blot assay.

Another homogenized suspension (including ocadeic acid) was directly incubated in 1% Triton x 100 to obtain whole heart samples containing the entire proteins of the myocyte. After 60 min of incubation on ice, the solution was vortexed at 12000 g for 2 min to clear the fraction up, and then stored at -80°C until further use.

Western blot analysis

After protein determination by the Lowry method(15), equal amounts of protein were mixed with loading buffer (1:1) containing Tris-HCl, glycerol and bromphenol blue. Samples were vortexed and boiled at 95°C before being subjected to SDS-PAGE. Samples were loaded on a 10% SDS electrophoresis gel. The proteins were separated by electrophoresis and transferred to a PVDF membrane by tank blotting (100V, 1h). Unspecific binding of the antibody was blocked by incubation with 5% fat dry milk powder in Tris buffered saline containing Tween (TBS-T) for 2 hours. Subsequently, the membrane was incubated over night at 4°C with the COX-2 or actin antibody, respectively, at indicated concentrations. After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands were visualized by chemiluminescence detected on X-ray film (Hyperfilm ECL, Amersham) using the enhanced chemiluminescence system Santa Cruz. The blots were quantified using a Kodak Image station® (Eastman Kodak Comp., Rochester, NY) and the results are presented as ratio of COX-2 to actin. Equal loading of the protein to the SDS Page gel was ensured by Coomassie blue staining (Coomassie brillant blue®, Serva electrohoresis GmbH, Heidelberg, Germany) of each gel. The results are presented as ratio of total protein to actin, which was used as internal standard.

Statistical Analysis

Data are expressed as mean ± SD. Heart rate (HR, in bpm) and mean aortic pressure (AOPmean, in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion period. Inter-group differences of hemodynamic data were analyzed (SPSS Science Software, version 12.0.1) by performing a One-way ANOVA followed by Tukey's post-hoc test. Time effects (changes from baseline value) during the experiments and infarct sizes were analyzed by using a One-way ANOVA followed by Dunnett's post-hoc test. Changes within and between groups were considered statistically significant if $p<0.05$.

RESULTS

Infarct size measurement

Infarct size was reduced by all three helium concentrations from 55 ± 8 % in controls (n = 8) to 37 ± 13 % in He-LPC 70 (n = 12), to 34 ± 16 % in He-LPC 50 (n = 7) and to 40 ± 9 % in He-LPC 30 (n = 7) (each $P<0.05$ vs. Con, fig. 2A). Based on these findings part 2 of the study was performed with 30% Helium. Daily administration of helium more than one day

did not further enhance cardioprotection (He-LPC 3x30 (n = 5): 39 ± 9 %, He-LPC 2x30 (n = 6): 38 ± 10%; P>0.05 vs. He-LPC 1x30 (n = 6): 37 ± 11%, fig. 2B).

Hemodynamic variables

Hemodynamic variables are summarized in table 1. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischemia or reperfusion. At the end of the experiments of part 1, mean aortic pressure significantly decreased compared with baseline in all groups. In part 2, mean aortic pressure did also decrease compared with baseline in all groups at the end of the experiments.

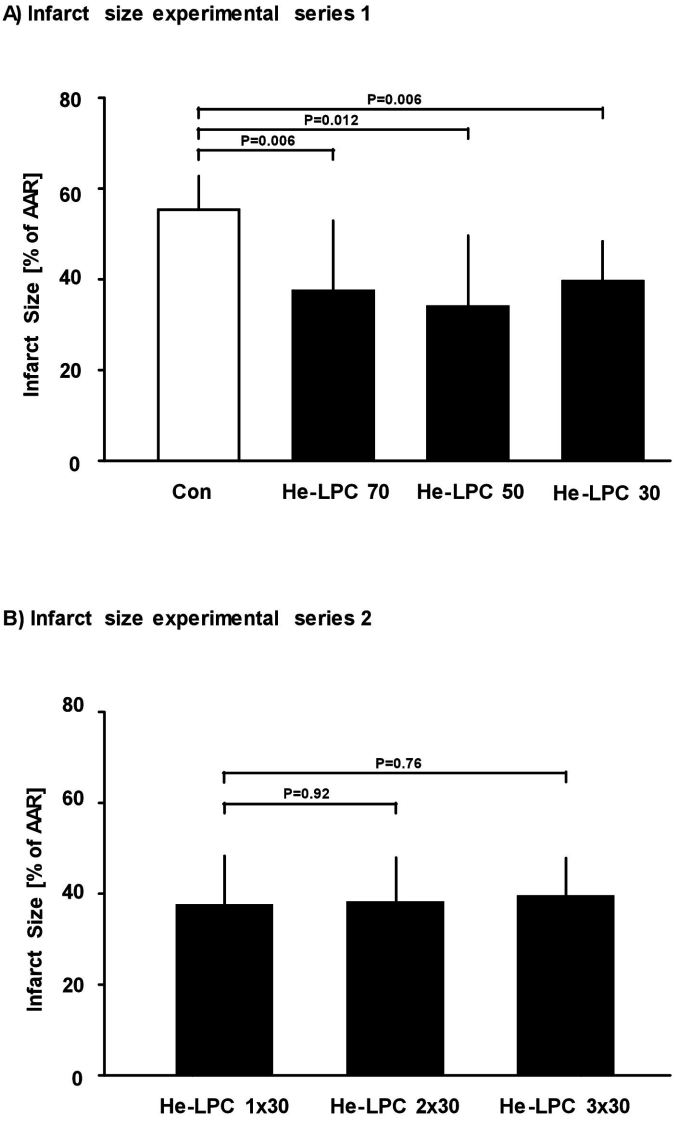
Mitochondrial function

The respiratory control indices are shown in figure 3. RCI in the control group was 2.8 ± 0.2. There were no significant changes in the RCI at 6 h, 10 h or 24 h after 30% helium administration (P>0.05 vs. Con, fig. 3).

Western blot analysis of COX-2

Helium pretreatment did not affect COX-2 expression neither in the cytosolic fraction (He-LPC 6h: 0.25 ± 0.07, He-LPC 10h: 0.27 ± 0.07, He-LPC 24h: 0.26 ± 0.06; P>0.05 vs Con: 0.22 ± 0.10, each n = 5, fig. 4A), nor in the membrane fraction (He-LPC 6h: 0.44 ± 0.26, He-LPC 10h: 0.35 ± 0.15, He-LPC 24h: 0.36 ± 0.06; P>0.05 vs Con: 0.54 ± 0.24, each n = 5, fig. 4B). Furthermore we analyzed COX-2 expression in the whole heart fraction, but could not detect any changes (He-LPC 6h: 1.32 ± 0.37, He-LPC 10h: 1.11 ± 0.27, He-LPC 24h: 1.11 ± 0.23; P>0.05 vs Con: 1.25 ± 0.37, each n = 5, fig. 4C).

Figure 2:



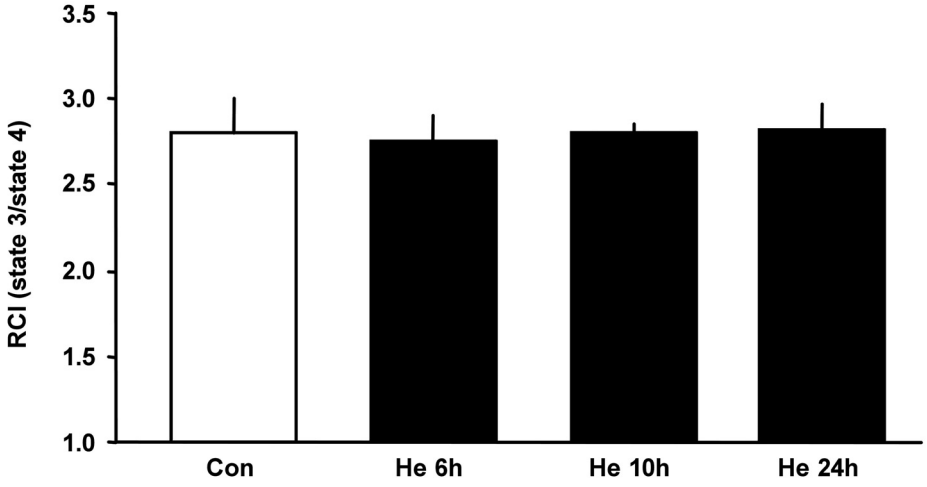
Panel A: Histogram showing the infarct size (percent of area at risk, AAR) of controls (Con), late preconditioning with 70% helium (He-LPC 70), late preconditioning with 50% helium (He-LPC 50) and late preconditioning with 30% helium (He-LPC 30). *Panel B:* Histogram showing the infarct size (percent of AAR), late preconditioning with three times 30% helium (He-LPC 3x30), two times 30% helium (He-LPC 2x30), and one time 30% helium (He-LPC 1x30).
Data are presented as mean ± SD, $*p < 0.05$ vs. control group.

Table 1: Hemodynamic variables

	Baseline	Ischemia	Reperfusion			
		15	5	30	60	120
Heart Rate (bpm)						
Con	421 ± 21	427 ± 24	416 ± 17	394 ± 18	387 ± 17	372 ± 28
He-LPC 70	414 ± 32	432 ± 30	407 ± 49	415 ± 28	406 ± 25	367 ± 36
He-LPC 50	419 ± 31	415 ± 28	414 ± 21	402 ± 28	383 ± 31	369 ± 37
He-LPC 30	411 ± 31	409 ± 37	409 ± 35	424 ± 33	404 ± 28	377 ± 35
He-LPC 1x30	396 ± 41	394 ± 35	390 ± 37	388 ± 32	378 ± 34	363 ± 44
He-LPC 2x30	407 ± 37	407 ± 31	399 ± 40	388 ± 41	391 ± 45	370 ± 44
He-LPC 3x30	413 ± 27	413 ± 38	405 ± 42	402 ± 38	388 ± 40	357 ± 29
Mean aortic pressure (mmHg)						
Con	129 ± 20	115 ± 25	118 ± 29	100 ± 31	90 ± 33	84 ± 28*
He-LPC 70	121 ± 19	122 ± 18	119 ± 21	110 ± 23	102 ± 27	92 ± 17*
He-LPC 50	118 ± 19	117 ± 24	118 ± 20	101 ± 20	91 ± 30	78 ± 26*
He-LPC 30	138 ± 11	139 ± 19	135 ± 22	117 ± 15	108 ± 28	88 ± 19*
He-LPC 1x30	137 ± 15	129 ± 17	117 ± 19	117 ± 18	102 ± 23*	95 ± 23*
He-LPC 2x30	135 ± 30	123 ± 20	112 ± 18	111 ± 17	110 ± 27	97 ± 22*
He-LPC 3x30	131 ± 23	118 ± 33	122 ± 33	103 ± 36	105 ± 27	90 ± 28

Data are mean ± SD. Con = control group; He-LPC 70 = Helium late preconditioning with 70% Helium + 30% oxygen; He-LPC 50 = Helium late preconditioning with 50% Helium + 30% oxygen + 20% nitrogen; He-LPC 30 = Helium late preconditioning with 30% Helium + 30% oxygen + 40% nitrogen;*P<0.05 vs. baseline.

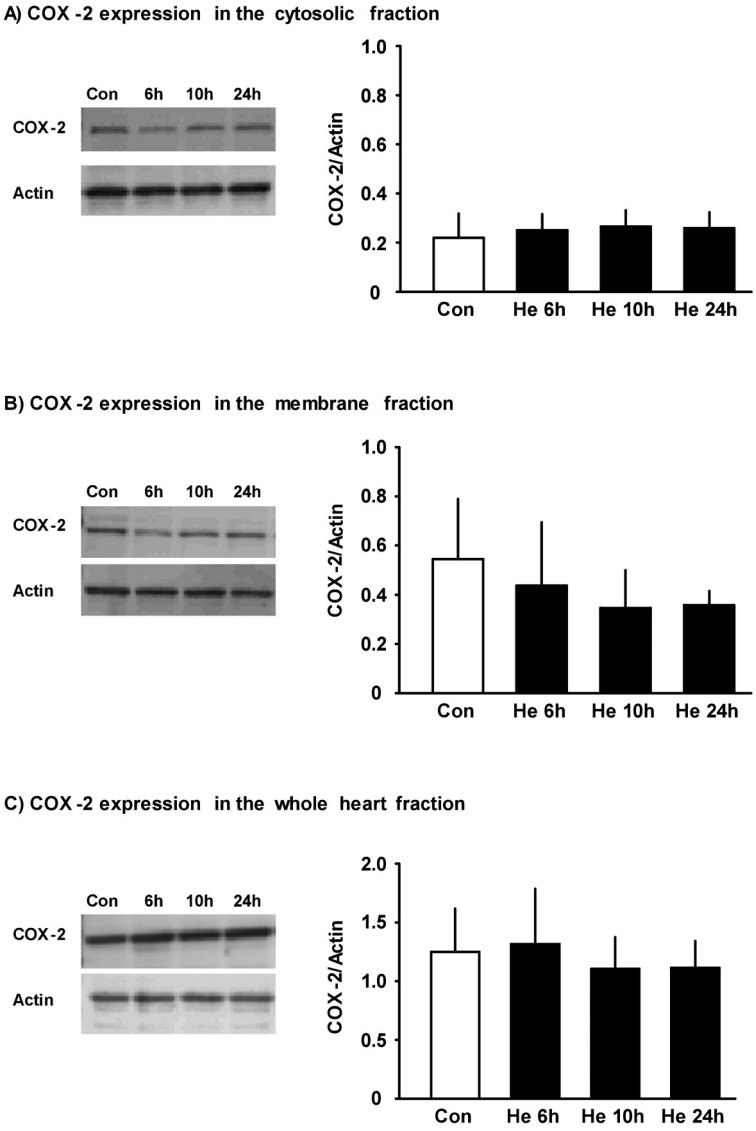
Figure 3: Mitochondrial respiration



state 3 (nmol O_2/mg/min)	256 ± 14	249 ± 24	255 ± 15	254 ± 6
state 4 (nmol O_2/mg/min)	91 ± 9	90 ± 11	91 ± 4	90 ± 3
P/O ratio	1.72 ± 0.05	1.69 ± 0.12	1.71 ± 0.05	1.73 ± 0.04

Summarized data for the effects of helium-induced late preconditioning on mitochondrial respiration (each n = 5, mean ± SD). RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into ATP and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation.

Figure 4:



Time dependent effect of helium-induced late preconditioning on COX-2 expression (each n = 5, mean ± SD). *Upper panel* shows time dependent expression of COX-2; *middle panel* shows actin distribution. The histogram presents ratio of COX-2 expression to actin. *Panel A*: COX-2 expression in the cytosolic fraction. *Panel B*: COX-2 expression in the membrane fraction. *Panel C*: COX-2 expression in the whole heart fraction.

DISCUSSION

The main results of the present study are: 1) helium induces late preconditioning *in vivo*, 2) one-time 30% helium induces cardioprotection in the same range as 50% or 70% helium, 3) repeated administration of helium can not further enhance cardioprotection and 4) helium LPC has no effect on COX-2 expression.

The late phase of preconditioning starts 12-24 hours after the preconditioning stimulus and lasts up to 4 days. (2) These properties would make LPC an interesting intervention, e.g. for patients with cardiac disease undergoing coronary angioplasty or cardiac bypass surgery. LPC would gain more importance, if it could be induced by a substance which is easy to administer and without any hemodynamic or anesthetic properties.

It is known that early preconditioning can be induced by administration of noble gases. A cardioprotective effect against reperfusion injury of the anesthetic noble gas xenon has previously been demonstrated. (23; 30) Pagel et al. recently showed that the noble gas helium offers protection against myocardial infarction by EPC. (22) In the present study we demonstrate for the first time that helium induces also LPC and reduces infarct size by nearly 40% compared to non-preconditioned myocardium. Interestingly, the extend of cardioprotection induced by 30% helium was comparable to the cardioprotection induced by 50% or 70% helium. The possibility to offer cardioprotection with less than 70% helium would allow to apply helium as preconditioning stimulus while having a high F_iO_2, e.g. when used in cardiac risk patients. In contrast to xenon, helium has no anesthetic effects, allowing a broader application. The use of xenon implies considerable costs, making special application systems necessary to limit costs. In contrast, helium is relatively cheap and easy to administer via a face mask, making it a suitable agent for organ protection in many clinical situations.

Baxter et al. showed that more than one preconditioning cycle on the same day 24 hours before I/R did not enhance the infarct size limiting effect of ischemic LPC. (1) A study by Mullenheim et al. showed that combining ischemic LPC and ischemic EPC led to enhanced cardioprotection compared to LPC or EPC alone. (20) The same group showed that cardioprotection by ischemic LPC could be increased by sevoflurane-induced EPC. (18) A study by Sato et al. suggested an additive cardioprotective effect by combining ischemic

LPC with ischemic postconditioning whereas each several intervention could not induce myocardial protection. (25) There is no study investigating the infarct size reducing effect of repeated preconditioning cycles administered on subsequent days. Our results demonstrate that repeated cycles of helium administration on subsequent days could not enhance cardioprotection within the time window of LPC.

Studies investigating LPC indicate that a crucial step in mediating cardioprotection involves COX-2. (4; 10; 27) Beside ischemic LPC, also pharmacological induced LPC led to an upregulation of COX-2 protein after 24 hours. (9; 26) Upregulation of COX-2 and its products, the prostaglandins PGE_2 and 6-keto-$PGF_{1\alpha}$ was observed in both, ischemic- and opioid-induced LPC. (26; 27) However, there are obviously differences in animal species: in rabbits and mice an upregulation of COX-2 was observed, whereas in rats, there was no upregulation of COX-2 after LPC. (10; 14; 26; 27) In the present study we did not detect any changes in COX-2 expression in the period up to 24 hours after helium preconditioning. We demonstrated previously by inhibiting COX-2 with the selective blocker NS-398 that COX-2 is involved in xenon-induced LPC. (8) A study by Tanaka et al. showed no alteration in COX-2 expression 24 hours after exposure to the volatile anesthetic isoflurane, but blockade of cardioprotection by using the COX-2 inhibitor celecoxib. (28) Our results indicate that COX-2 protein expression is not affected by exposure to helium up to 24 hours before I/R. However, we cannot exclude that the LPC effect of helium is due to an increase of COX-2 activity because it was not measured in the current study.

Pagel et al. showed that helium induced EPC in the rabbit heart *in vivo* is abolished by inhibiting phosphatidylinositol-3-kinase, extracellular signal-regulated kinase and 70-kDa ribosomal protein s6 kinase. (22) Furthermore, they demonstrated that cardioprotection was abolished by administration of the mPTP opener atractyloside, indicating a central role of mPTP in helium-induced early preconditioning. (22) Alongside its role in early preconditioning, there is evidence that prevention of mPTP opening is also involved in late preconditioning. (24) Rajesh et al. showed that the protective effect of delayed ischemic preconditioning was abolished by administration of the mPTP opener atractyloside or lonidamine. (24) Opening of the mPTP can be regulated by alterations in mitochondrial bioenergetics, e.g. mild uncoupling of mitochondrial respiration. (11) In the present study, we did not detect a reduction in the RCI 6h, 10h, and 24h after helium administration

indicating that alterations in mitochondrial respiration are not involved in helium-induced LPC.

Some limitations of our study must be taken into account. First, we did not investigate the effect of various cycles of helium administration on the same day 24 hours before I/R. It might be possible that repeated cycles of helium on the same day could enhance the cardioprotective effect. Second, in this study we restricted the measurement of biochemical markers to the time course of COX-2. Conclusions about the involvement of other candidate mediators like iNOS need further study.

In conclusion, the current study demonstrates that inhalation of one-time 30% helium 24 hours before I/R offers cardioprotection in the same range as reported by other studies investigating pharmacological induced LPC. The cardioprotective effect was not associated with an increased COX-2 expression or an uncoupling of mitochondrial respiration.

REFERENCES

1. **Baxter GF, Goma FM and Yellon DM**. Characterisation of the infarct-limiting effect of delayed preconditioning: timecourse and dose-dependency studies in rabbit myocardium. *Basic Res Cardiol* 92: 159-167, 1997.
2. **Bolli R**. The late phase of preconditioning. *Circ Res* 87: 972-983, 2000.
3. **Bolli R, Dawn B, Tang XL, Qiu Y, Ping P, Xuan YT, Jones WK, Takano H, Guo Y and Zhang J**. The nitric oxide hypothesis of late preconditioning. *Basic Res Cardiol* 93: 325-338, 1998.
4. **Bolli R, Shinmura K, Tang XL, Kodani E, Xuan YT, Guo Y and Dawn B**. Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res* 55: 506-519, 2002.
5. **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
6. **Chen Y, Rajashree R, Liu Q and Hofmann P**. Acute p38 MAPK activation decreases force development in ventricular myocytes. *Am J Physiol Heart Circ Physiol* 285: H2578-H2586, 2003.
7. **Chiari PC, Pagel PS, Tanaka K, Krolikowski JG, Ludwig LM, Trillo RA, Jr., Puri N, Kersten JR and Warltier DC**. Intravenous emulsified halogenated anesthetics produce acute and delayed preconditioning against myocardial infarction in rabbits. *Anesthesiology* 101: 1160-1166, 2004.
8. **Frassdorf J, Weber NC, Ratajczak C, Schlack W and Preckel B**. Xenon induces late preconditioning in rat heart in vivo - involvement of COX-II [abstract]. *Eur J Anaesthesiol* 23 Supplement 37: A-2, 2006.
9. **Fryer RM, Hsu AK, Eells JT, Nagase H and Gross GJ**. Opioid-induced second window of cardioprotection: potential role of mitochondrial K_{ATP} channels. *Circ Res* 84: 846-851, 1999.

10. **Guo Y, Bao W, Wu WJ, Shinmura K, Tang XL and Bolli R**. Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol* 95: 479-484, 2000.
11. **Halestrap AP, Clarke SJ and Khaliulin I**. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 1767: 1007-1031, 2007.
12. **Heinen A, Winning A, Schlack W, Hollmann MW, Preckel B, Fräßdorf J and Weber NC**. The regulation of mitochondrial respiration by opening of mK_{Ca} channels is age-dependent. *Eur J Pharmacol* 578: 108-113, 2008.
13. **Kang N, Alexander G, Park JK, Maasch C, Buchwalow I, Luft FC and Haller H**. Differential expression of protein kinase C isoforms in streptozotocin-induced diabetic rats. *Kidney Int* 56: 1737-1750, 1999.
14. **Kodani E, Xuan YT, Shinmura K, Takano H, Tang XL and Bolli R**. Delta-opioid receptor-induced late preconditioning is mediated by cyclooxygenase-2 in conscious rabbits. *Am J Physiol Heart Circ Physiol* 283: H1943-H1957, 2002.
15. **Lowry OH, Rosebrough NJ, Farr AL and Randall RJ**. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
16. **MacKay K and Mochly-Rosen D**. Arachidonic acid protects neonatal rat cardiac myocytes from ischaemic injury through epsilon protein kinase C. *Cardiovasc Res* 50: 65-74, 2001.
17. **McPherson BC and Yao Z**. Morphine mimics preconditioning via free radical signals and mitochondrial K_{ATP} channels in myocytes. *Circulation* 103: 290-295, 2001.
18. **Mullenheim J, Ebel D, Bauer M, Otto F, Heinen A, Frassdorf J, Preckel B and Schlack W**. Sevoflurane confers additional cardioprotection after ischemic late preconditioning in rabbits. *Anesthesiology* 99: 624-631, 2003.
19. **Mullenheim J, Frassdorf J, Preckel B, Thamer V and Schlack W**. Ketamine, but not S(+)-ketamine, blocks ischemic preconditioning in rabbit hearts in vivo. *Anesthesiology* 94: 630-636, 2001.
20. **Mullenheim J, Schlack W, Frassdorf J, Heinen A, Preckel B and Thamer V**. Additive protective effects of late and early ischaemic preconditioning are mediated by the opening of K_{ATP} channels in vivo. *Pflugers Arch* 442: 178-187, 2001.
21. **Obal D, Preckel B, Scharbatke H, Mullenheim J, Hoterkes F, Thamer V and Schlack W**. One MAC of sevoflurane provides protection against reperfusion injury in the rat heart in vivo. *Br J Anaesth* 87: 905-911, 2001.
22. **Pagel PS, Krolikowski JG, Shim YH, Venkatapuram S, Kersten JR, Weihrauch D, Warltier DC and Pratt PF, Jr.** Noble gases without anesthetic properties protect myocardium against infarction by activating prosurvival signaling kinases and inhibiting mitochondrial permeability transition in vivo. *Anesth Analg* 105: 562-569, 2007.
23. **Preckel B, Mullenheim J, Moloschavij A, Thamer V and Schlack W**. Xenon administration during early reperfusion reduces infarct size after regional ischemia in the rabbit heart in vivo. *Anesth Analg* 91: 1327-1332, 2000.
24. **Rajesh KG, Sasaguri S, Zhitian Z, Suzuki R, Asakai R and Maeda H**. Second window of ischemic preconditioning regulates mitochondrial permeability transition pore by enhancing Bcl-2 expression. *Cardiovasc Res* 59: 297-307, 2003.
25. **Sato H, Bolli R, Rokosh GD, Bi Q, Dai S, Shirk G and Tang XL**. The cardioprotection of the late phase of ischemic preconditioning is enhanced by postconditioning via a COX-2-mediated mechanism in conscious rats. *Am J Physiol Heart Circ Physiol* 293: H2557-H2564, 2007.
26. **Shinmura K, Nagai M, Tamaki K, Tani M and Bolli R**. COX-2-derived prostacyclin mediates opioid-induced late phase of preconditioning in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 283: H2534-H2543, 2002.

27. **Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A and Bolli R**. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A* 97: 10197-10202, 2000.
28. **Tanaka K, Ludwig LM, Krolikowski JG, Alcindor D, Pratt PF, Kersten JR, Pagel PS and Warltier DC**. Isoflurane produces delayed preconditioning against myocardial ischemia and reperfusion injury: role of cyclooxygenase-2. *Anesthesiology* 100: 525-531, 2004.
29. **Toma O, Weber NC, Wolter JI, Obal D, Preckel B and Schlack W**. Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart in vivo. *Anesthesiology* 101: 1372-1380, 2004.
30. **Weber NC, Toma O, Wolter JI, Obal D, Mullenheim J, Preckel B and Schlack W**. The noble gas xenon induces pharmacological preconditioning in the rat heart in vivo via induction of PKC-epsilon and p38 MAPK. *Br J Pharmacol* 144: 123-132, 2005.

Chapter 8

Hyperglycaemia blocks Sevoflurane-induced postconditioning in the rat heart *in vivo*: Cardioprotection can be restored by blocking the mitochondrial permeability transition pore

Ragnar Huhn, André Heinen, Nina C. Weber, Markus W. Hollmann, Wolfgang Schlack, Benedikt Preckel

British Journal of Anaesthesia
2008 Apr;100(4):465-71

ABSTRACT

Recent studies showed that hyperglycaemia (HG) blocks anaesthetic-induced preconditioning. The influence of HG on anaesthetic-induced postconditioning (post) has not yet been determined. We investigated whether sevoflurane (Sevo)-induced postconditioning is blocked by HG and whether the blockade could be reversed by inhibiting the mitochondrial permeability transition pore (mPTP) with cyclosporine A (CsA). Chloralose-anaesthetized rats (n=7–11 per group) were subjected to 25 min coronary artery occlusion followed by 120 min reperfusion. Postconditioning was achieved by administration of 1 or 2 MAC sevoflurane for the first 5 min of early reperfusion. HG was induced by infusion of glucose 50% (G 50) for 35 min, starting 5 min before ischaemia up to 5 min of reperfusion. CsA (5 or 10 mg kg^{-1}) was administered i.v. 5 min before the onset of reperfusion. At the end of the experiments, hearts were excised for infarct size measurements. Infarct size (% of area at risk) was reduced from 51.4 (5.0)% [mean (SD)] in controls to 32.7 (12.8)% after sevoflurane postconditioning (Sevo-post) (P<0.05). This infarct size reduction was completely abolished by HG [51.1 (13.2)%, P<0.05 *vs* Sevo-post], but was restored by administration of sevoflurane with CsA [35.2 (5.2)%, P<0.05 *vs* HG+Sevo-post]. Increased concentrations of sevoflurane or CsA alone could not restore cardioprotection in a state of HG [Sevo-post2, 54.1 (12.6)%, P>0.05 *vs* HG+Sevo-post; CsA10, 58.8 (11.3)%, P>0.05 *vs* HG+CsA]. Sevoflurane-induced postconditioning is blocked by HG. Inhibition of the mPTP with CsA is able to reverse this loss of cardioprotection

INTRODUCTION

Hyperglycaemia correlates with increased mortality after acute myocardial infarction in diabetic patients as well as in patients without diabetes mellitus. (2; 13) Hyperglycaemia was shown to abolish cardioprotection induced by ischaemic and anaesthetic preconditioning. (14; 15)

Besides *pre*conditioning, also *post*conditioning (i.e. cardioprotection by administration of the substance after ischaemia during early reperfusion) can be induced by volatile anaesthetics. (3; 23) Recent studies demonstrated, that the volatile anaesthetic sevoflurane offers cardioprotection by postconditioning. (5; 21) In both studies, postconditioning induced a cardioprotective effect that was comparable to the extent of cardioprotection induced by sevoflurane preconditioning. Furthermore, Obal et al. showed that sevoflurane induces maximal cardioprotection by postconditioning at a concentration of only 1 MAC. (22) It is not known whether anaesthetic-induced postconditioning can be induced in hyperglycaemic subjects. This question was tested in the first part of the study using the *in vivo* rat model.

Recent studies showed that the mPTP is involved in isoflurane-induced postconditioning via phosphorylation and inhibition of GSK3β. (8) Krolikowski et al. demonstrated that keeping the mPTP closed with CsA enhanced cardioprotection of isoflurane-induced postconditioning. (17) Therefore, in the second part of the study, we tested if administration of CsA shortly before the reperfusion period could restore the assumed abolished cardioprotection.

We hypothesized that (1) sevoflurane postconditioning is abolished by hyperglycaemia and (2) that the cardioprotection can be restored by inhibiting the mPTP in hyperglycaemic animals.

MATERIALS AND METHODS

The study was performed in accordance with the requirements of the Animal Ethics Committee of the University of Amsterdam and was in line with European Union directives on the care and use of experimental animals.

Materials

Sevoflurane was purchased from Abbott (SEVOrane®, Abbott B.V., Hoofddorp, the Netherlands). Cyclosporine A was purchased from Fluka Biochemika (Sigma Aldrich, Steinheim, Germany). The Glucose 50% was purchased from B. Braun (B. Braun Melsungen AG, Melsungen, Germany). For measurement of the blood glucose levels, we used the FreeStyle Freedom blood glucose meter from Abbott. Rat insulin samples were measured with a Rat Insulin ELISA from Orange Medical (Orange Medical, Tilburg, the Netherlands).

Experimental protocol for infarct size determination:

Animals had free access to food and water at all times before the start of the experiments. Male Wistar rats (250-350 g, 7-11 per group) were anaesthetized by intraperitoneal S-ketamine injection (150 mg kg^{-1}). S-ketamine does not interfere with cardioprotection in animals in vivo. (19)

Rats were divided into ten groups (Fig. 1A):

All animals underwent 25 min of coronary artery occlusion and 2 hours of reperfusion (I/R).

Control group (Con) (n = 9): After surgical preparation, rats received 30% oxygen plus 70% nitrogen. Normal saline was given intravenously over 35 minutes starting 5 min prior ischaemia up to 5 minutes of reperfusion.

Sevoflurane postconditioned group (Sevo-post) (n = 11): Rats received sevoflurane with an endtidal concentration of 1 MAC (≙ 2.4 Vol%) for 5 minutes starting 1 minute prior to the onset of reperfusion; saline 0.9% was infused intravenously over 35 minutes starting 5 min prior ischaemia up to 5 minutes of reperfusion.

Glucose 50% group (HG) (n = 9): Glucose 50% was administered intravenously over 35 minutes starting 5 minutes prior to ischaemia and was continued until 5 minutes of reperfusion. Target blood glucose level before ischaemia was 22 mmol l^{-1} or higher and was maintained at this level.

Glucose 50% + Sevoflurane postconditioned group (HG+Sevo-post) (n = 9): glucose 50% and sevoflurane were both given as described above.

CsA group (CsA) (n = 9): CsA (5 mg kg^{-1} in DMSO 1% aqueous solution)(17) was administered intravenously 5 minutes before reperfusion; saline 0.9% was infused intravenously over 35 minutes starting 5 min prior ischaemia up to 5 minutes of reperfusion.

CsA + Sevoflurane postconditioned group (CsA+Sevo-post) (n = 8): rats received CsA and sevoflurane as described above.

Glucose 50% + CsA group (HG+CsA) (n = 8): Rats received Glucose 50% and CsA (5 mg kg^{-1}) intravenously as described above.

Glucose 50% + CsA + Sevoflurane postconditioned group (HG+CsA+Sevo-post) (n = 8): rats received glucose 50%, CsA (5 mg kg^{-1}) intravenously, and inhaled sevoflurane as described above.

To investigate whether a higher concentration of sevoflurane or CsA alone could restore cardioprotection in state of hyperglycaemic condition we added two more groups with 2 MAC sevoflurane and 10 mg kg^{-1} CsA:

Glucose 50% + Sevoflurane postconditioned group (HG+Sevo-post2) (n = 9): glucose 50% and 2 MAC sevoflurane were both given as described above.

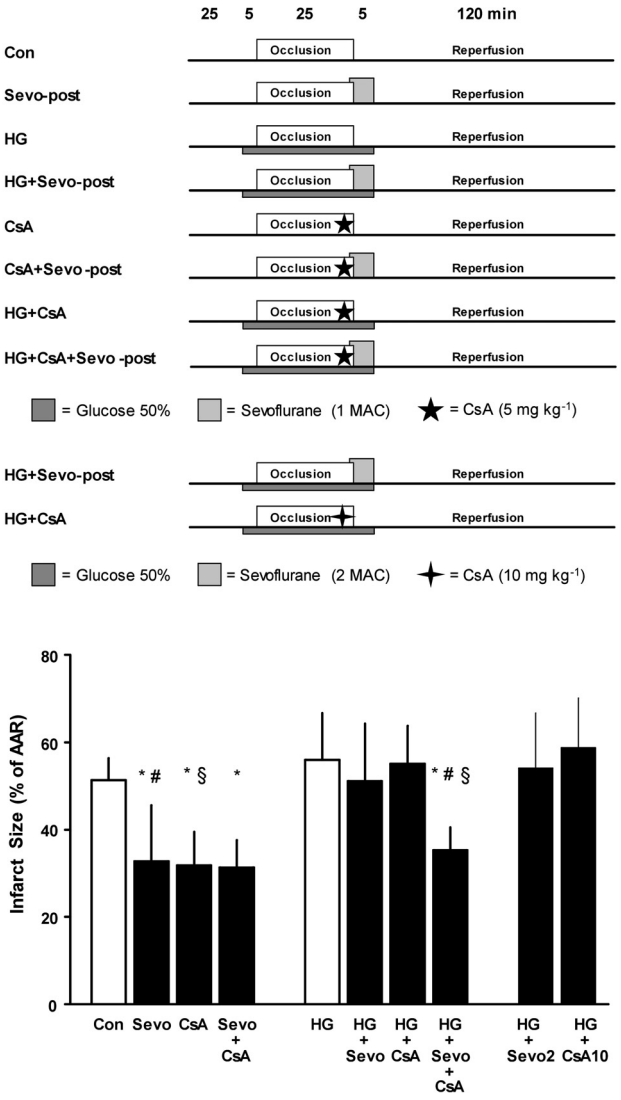
Glucose 50% + CsA group (HG+CsA10) (n = 7): Rats received Glucose 50% and CsA (10 mg kg^{-1}) intravenously as described above.

Surgical preparation and infarct size measurement:

Surgical preparation was performed as described previously. (22; 24) In brief, male Wistar rats (250-350 g) were anaesthetized by intraperitoneal S-ketamine injection (150 mg kg^{-1}). Respiratory rate was adjusted to maintain PCO_2 within physiological limits. Body temperature was maintained at 38°C by the use of a heating pad. Anaesthesia was maintained by continuous α-chloralose infusion. A lateral left sided thoracotomy followed by pericardiotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery. All animals were left untreated for 25 minutes before the start of the respective experimental protocol. Arterial blood gases were analyzed at baseline and PCO_2 and PO_2 were kept within physiological ranges by adjusting ventilation. Sevoflurane concentration was measured in the expiratory gas (Datex Capnomac Ultima, Division of Instrumentarium Corp., Helsinki, Finland). Aortic pressure and electrocardiographic signals were digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments).

After 120 minutes of reperfusion, the heart was excised and infarct size was determined as previously described. (22) The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight of each slice.

Figure 1:



(A) Experimental protocol. Sevo, sevoflurane; post, postconditioning; HG, hyperglycaemia; CsA, cyclosporine A. (B) Infarct size measurement. Histogram shows the infarct size (per cent of area at risk, AAR) of controls (Con), sevoflurane postconditioning (Sevo), hyperglycaemia (HG) alone, hyperglycaemia and sevoflurane postconditioning (HG+Sevo), cyclosporine A (CsA) alone, cyclosporine A and sevoflurane postconditioning (CsA+Sevo), hyperglycaemia and cyclosporine A (HG+CsA), hyperglycaemia and cyclosporine A and sevoflurane postconditioning (HG+CsA+Sevo), hyperglycaemia and sevoflurane postconditioning with 2 MAC (HG+Sevo2), hyperglycaemia and cyclosporine A with 10 mg kg^{-1} (HG+CsA10). Data shown are mean (SD). $^{*}P$<0.05 *vs* control group; $^{\#}P$<0.05 *vs* HG+Sevo; $^{\S}P$<0.05 *vs* HG+CsA (n = 7–11 per group).

Blood glucose and insulin measurement

Blood samples were collected at different times to measure blood glucose in each group (see Tab. 1). Insulin levels were measured in order to determine a physiological endocrinal reaction to hyperglycaemia. Samples were taken before ischaemia, during ischaemia and after 30 min of reperfusion. During ischaemia, insulin level was 4 fold increased in the hyperglycaemic groups compared to non hyperglycaemic groups. After 30 min of reperfusion, insulin level was still 7 fold higher in the hyperglycaemic groups compared with the non hyperglycaemic groups.

Statistical Analysis

Data are expressed as mean (SD). Heart rate (HR, in bpm) and mean aortic pressure (AOPmean, in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion period. Inter-group differences of haemodynamic data were analyzed (SPSS Science Software, version 12.0.1) by performing a One-way ANOVA followed by Tukey's post-hoc test. Time effects (changes from baseline value) during the experiments were analyzed by using a One-way ANOVA followed by Dunnett's post-hoc test. Infarct sizes were analyzed by a One-way ANOVA followed by Tukey's post-hoc test. Changes within and between groups were considered statistically significant if $p<0.05$.

RESULTS

Blood glucose measurement

Glucose levels during the experimental protocol are shown in table 1.

Mean baseline blood glucose levels were 7.1 (1.2) mmol l^{-1} and did not differ between the groups. Before ischaemia, mean blood glucose levels were 25.5 (1.5) mmol l^{-1} in the hyperglycaemic groups and 7.1 (0.7) mmol l^{-1} in the non-hyperglycaemic groups. During ischaemia, blood glucose levels remained high at 26.6 (1.2) mmol l^{-1} in hyperglycaemic groups, while blood glucose was 7.0 (0.6) mmol l^{-1} in the non-hyperglycaemic groups. After 5 minutes of reperfusion, blood glucose levels were 26.0 (1.3) mmol l^{-1} in the hyperglycaemic groups and then declined to 5.9 (0.9) mmol l^{-1} at the end of the reperfusion period. In the non-hyperglycaemic groups, blood glucose levels were 6.8 (0.5) mmol l^{-1} after 5 minutes of reperfusion and declined to 4.9 (0.6) mmol l^{-1} at the end of the reperfusion period. Blood glucose levels at the end of the reperfusion period of the non-hyperglycaemic groups were significantly decreased compared to baseline. In contrast, no

significant differences were found in hyperglycaemic groups when comparing baseline values to values at the end of reperfusion.

Table 1:

	Baseline		Ischaemia	Reperfusion		
	5	30	15	5	30	120
Con	7.7 (2.1)	7.9 (2.2)	7.5 (2.4)	7.1 (1.5)	5.8 (1.0)	4.4 (0.9)*
Sevo-post	7.8 (1.2)	7.4 (1.2)	7.3 (1.3)	7.3 (1.5)	6.5 (0.9)	5.8 (0.6)*
HG	7.6 (1.8)	24.9 (2.3)	26.5 (0.9)	25.1 (1.4)	11.7 (3.1)	5.8 (1.2)
HG+Sevo-post	8.4 (1.4)	24.7 (2.3)	27.0 (1.3)	25.8 (1.1)	13.4 (3.4)	6.9 (1.0)
CsA	6.8 (1.0)	6.2 (0.6)	6.3 (0.8)	6.4 (1.0)	5.2 (0.7)	4.8 (0.6)*
CsA+Sevo-post	6.7 (1.3)	6.7 (1.4)	6.3 (0.9)	6.4 (1.3)	5.1 (0.5)	4.6 (0.4)*
HG+CsA	7.1 (0.8)	24.5 (0.9)	25.9 (1.4)	25.6 (1.2)	11.6 (3.6)	5.3 (0.7)
HG+CsA+Sevo-post	6.2 (0.4)	24.8 (0.7)	27.2 (0.6)	26.3 (1.7)	11.6 (2.6)	6.0 (1.3)
HG+Sevo-post2	6.5 (0.7)	27.1 (1.4)	26.5 (0.9)	26.8 (0.9)	15.1 (2.0)	5.6 (0.6)
HG+CsA10	6.7 (0.7)	27.4 (1.1)	26.2 (1.8)	26.3 (1.5)	13.9 (3.4)	5.9 (0.6)

Blood glucose values (mmol litre^{-1}). Data are mean (SD). Con, control; Sevo-post, 1 MAC sevoflurane postconditioning; Sevo-post2, 2 MAC sevoflurane postconditioning; HG, hyperglycaemia; CsA, cyclosporine A 5 mg kg^{-1}; CsA10, cyclosporine A 10 mg kg^{-1}. *P<0.05 *vs* baseline

Haemodynamic variables

Haemodynamic variables are summarized in table 2. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline. In sevoflurane treated groups, mean aortic pressure was transiently reduced during the postconditioning period with exception of the CsA+Sevo-post group. At the end of the experiments, mean aortic pressure was significantly decreased compared with baseline in all groups with the exception of the hyperglycaemic group.

Infarct size measurement

Infarct size was reduced from 51.4 (5.0)% in controls to 32.7 (12.8)% after sevoflurane postconditioning ($p<0.05$, Fig. 1B). Hyperglycaemia alone had no effect on infarct size (56.0 (10.7)%) but abolished the postconditioning effect of sevoflurane (51.1 (13.2)%, $p<0.05$ vs. Sevo-post). In normoglycaemic rats, CsA had a similar infarct reducing effect as sevoflurane (31.8 (7.7)%), but combination of both drugs did not further reduce infarct size (31.3 (6.3)%, $p<0.05$ vs. controls). The cardioprotective effect of CsA alone was also

blocked by hyperglycaemia (55.0 (8.7)%, p>0.05 vs. controls). However, combination of CsA and Sevo provided the infarct sparing effect against hyperglycaemia (35.2 (5.2)%, p<0.05 vs. HG+Sevo-post respectively HG+CsA). Increasing the sevoflurane concentration to 2 MAC with hyperglycaemia (54.1 (12.6)%, p>0.05 vs. HG+Sevo-post) or CsA to 10 mg kg^{-1} CsA (58.8 (11.3)%, p>0.05 vs. HG+CsA) had no effect on infarct size.

Table 2:

	Baseline		Ischaemia	Reperfusion		
	5	25	15	5	30	120
HR (BPM)						
Con	431(21)	432(21)	430(31)	425(27)	419(17)	380(28)*
Sevo-post	421(61)	411(88)	444(27)	388(15)	422(42)	405(33)
HG	423(45)	432(15)	420(27)	399(24)	391(34)	357(29)*
HG+Sevo-post	426(55)	423(32)	415(53)	365(35)#	401(46)	410(25)
CsA	416(40)	418(44)	409(54)	421(39)	403(31)	397(30)
CsA+Sevo-post	402(19)	406(30)	397(33)	362(35)#	372(18)	359(26)
HG+CsA	418(42)	399(32)	414(42)	389(40)	392(30)	405(34)
HG+CsA+Sevo-post	428(46)	435(24)	413(61)	380(21)	413(35)	384(35)
HG+Sevo-post2	412(29)	400(29)	398(31)	344(21)#*	376(14)*	340(22)*
HG+CsA10	405(25)	389(24)	392(16)	392(35)	373(16)	339(17)*
AOPmean (mmHg)						
Con	127(21)	124(22)	111(20)	101(19)	101(26)	72(24)*
Sevo-post	140(17)	139(18)	122(34)	76(19)*	113(24)	98(20)*
HG	135(19)	144(17)	126(34)	106(33)	100(28)	93(48)
HG+Sevo-post	141(16)	148(28)	129(35)	74(23)*	104(36)	95(21)*
CsA	136(15)	123(18)	118(15)	133(12)	136(13)	96(20)*
CsA+Sevo-post	126(13)	125(19)	114(22)	99(28)	135(24)	87(17)*
HG+CsA	135(20)	145(19)	134(15)	124(20)	128(16)	99(19)*
HG+CsA+Sevo-post	136(14)	148(16)	119(22)	72(12)*	113(25)	82(28)*
HG+Sevo-post2	136(16)	144(16)	133(15)	74(12)*	103(18)	90(17)*
HG+CsA10	143(10)	149(12)	131(10)	124(31)	116(35)	79(22)*

Haemodynamic variables. Data are mean (SD). Con, control; Sevo-post, 1 MAC sevoflurane postconditioning; Sevo-post2, 2 MAC sevoflurane postconditioning; HG, hyperglycaemia; CsA, cyclosporine A 5 mg kg^{-1}; CsA10, cyclosporine A 10 mg kg^{-1}. *P<0.05 *vs* baseline; #P<0.05 *vs* control group

DISCUSSION

In the present study we investigated the effects of sevoflurane-induced postconditioning during hyperglycaemia. The main results show that: 1) hyperglycaemia abolishes cardioprotection by sevoflurane postconditioning and that 2) inhibition of mPTP with CsA reverses this loss of cardioprotection.

Diabetic and also hyperglycaemic non-diabetic patients with myocardial ischaemia-reperfusion like infarction or cardiac surgery, have a poorer prognosis than non diabetic or normoglycaemic controls. (2; 18) It is hypothetized that hyperglycaemia might cause a loss of (endogenous) cardioprotective mechanisms.

Beside cardioprotection by preconditioning, also cardioprotection by postconditioning, can be induced by ischaemic and pharmacological stimulie. (26) The protective effects of early as well as late preconditioning can be blocked by hyperglycaemia or diabetes mellitus. (7; 15) For ischaemic preconditioning it was shown that diabetes and hyperglycaemia of 17 and 34 mmol l^{-1} blocked cardioprotection *in vivo*. (15) The blockade was independent of plasma insulin concentrations and plasma osmolality. (16) Another study showed that, isoflurane-induced preconditioning was blocked by hyperglycaemia. (14) So far, there is no study available investigating the influence of hyperglycaemia on postconditioning. Postconditioning describes a cardioprotective intervention at the onset of myocardial reperfusion. In our study, postconditioning by sevoflurane reduced infarct size by nearly 40%, but sevoflurane postconditioning was abolished in state of hyperglycaemia.

For the hyperglycaemic groups we chose a blood glucose target level from 22 mmol l^{-1}. From a former study we know that this blood glucose concentration blocks desflurane-induced preconditioning. (6) Blood glucose levels used in the literature investigating the effect of hyperglycaemia on ischaemic- and isoflurane-induced preconditioning are quite in the same range. (14; 15) In our study blood glucose levels declined significantly at the end of the reperfusion period compared to baseline in the control group. Furthermore, this is the fact in all non-hyperglycaemic groups. There are two possible explanations for this blood glucose decrease: first, after preparation the animals were in a slight hyperglycaemic condition because of surgical stress and, second, the animals did not receive any substrates (e.g. glucose, free fatty acids) over the whole experimental protocol and reached normoglycaemic levels at the end of the experiments.

The opening of the mPTP occurs in the early minutes of reperfusion and is associated with the pathogenesis of necrosis and apoptosis. mPTP-opening might thus be regarded as a crucial step from reversible to irreversible cell death.(4) Inhibition of mPTP with CsA at the

onset of reperfusion was shown to protect the myocardium. (1; 11) In addition, it was demonstrated that 0.5 MAC isoflurane combined with CsA 5 mg kg^{-1} induced postconditioning, while 0.5 MAC isoflurane or CsA 5 mg kg^{-1} alone could not induce cardioprotection. In contrast, application of 1 MAC isoflurane or CsA at a dosage of 10 mg kg^{-1} were able to induce postconditioning. (17) In the current investigation, we used CsA in a concentration of 5 mg kg^{-1}. In our study, this low dosage led to a strong cardioprotection in rats in vivo. In two additional groups, 2 MAC sevoflurane alone or 10 mg kg^{-1} CsA alone in the hyperglycaemic condition were studied in order to investigate if higher doses of these agents given alone could restore cardioprotection. The data show that a single substance even at higher concentrations had no protective effect, in contrast to combination of the two substances (see figure 1).

A study by Chiari et al. showed that a non protective intervention with three times 10 seconds of ischaemic postconditioning, was enhanced by additional administration of 0.5 MAC isoflurane, a dosage which itself was also not protective. (3) These studies indicate that a triggered cardioprotective intervention with a non protective stimulus, a stimulus which does not confer cardioprotection by its own, could be enhanced by a second stimulus, assuming that there exist different and/or parallel cardioprotective pathways which could alter myocardial infarct size by various activation. The cited studies did not combine the two single protective interventions and the studies were not performed in hyperglycaemic animals. In our study, the combination of the two protective stimuli, 1 MAC Sevo and CsA did not result in enhanced cardioprotection in normoglycaemic animals. To our knowledge there is no study available showing that two protective stimuli by the same cardioprotective intervention, in this case postconditioning, could enhance the cardioprotective effect significantly in comparison to the single intervention. With regard to the combination of two different cardioprotective interventions, i.e. combination of ischaemic late preconditioning and early ischaemic preconditioning or early preconditioning and postconditioning, the literature is ambiguous. (5; 20) Our present results show that during hyperglycaemia, 1 MAC Sevo or 5 mg kg^{-1} CsA alone were not protective, but combination of both stimuli resulted in the full cardioprotective effect as observed in non-hyperglycaemic animals. Enhancement of the doses of sevoflurane to 2 MAC or CsA to a concentration of 10 mg kg^{-1} had no effect on infarct size in state of hyperglycaemia when the substances were given alone.

Elucidation of the molecular mechanisms involved in this cardioprotective interaction during hyperglycaemia is beyond the scope of the present study. The signal transduction pathways described for pharmacological postconditioning so far include i.e. PI3K/Akt,

MEK1/2, ERK1/2 and eNOS. (10; 12; 26) The signal transduction cascade consists of two parallel ways. Activation of PI3K/Akt leads to inhibition of the mPTP, whereas MEK1/2 via ERK1/2 activation finally leads to protein translation. (25) Both pathways interact with each other. The inhibition of the mPTP with CsA occurs downstream in the cascade of pharmacological postconditioning. (9) We speculate that sevoflurane amplifies the inhibition of the mPTP by CsA and additionally activates protein translation via a parallel pathway of the postconditioning cascade. Another explanation could be that the sole cardioprotective intervention with CsA or sevoflurane is not strong enough to protect the hyperglycaemic myocardium, but possibly the threshold for cardioprotection is lowered after combination of both protective pathways. Further research is needed to elucidate the molecular mechanisms contributing to this cardioprotective effect.

In summary, we demonstrated that hyperglycaemia blocks cardioprotection by sevoflurane-induced postconditioning, and that this loss of cardioprotection can be restored by CsA administration briefly before the onset of reperfusion.

REFERENCES

1. **Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J, Robert D and Ovize M**. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. *J Mol Cell Cardiol* 38: 367-374, 2005.
2. **Bellodi G, Manicardi V, Malavasi V, Veneri L, Bernini G, Bossini P, Distefano S, Magnanini G, Muratori L, Rossi G and .** Hyperglycemia and prognosis of acute myocardial infarction in patients without diabetes mellitus. *Am J Cardiol* 64: 885-888, 1989.
3. **Chiari PC, Bienengraeber MW, Pagel PS, Krolikowski JG, Kersten JR and Warltier DC**. Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: evidence for anesthetic-induced postconditioning in rabbits. *Anesthesiology* 102: 102-109, 2005.
4. **Crompton M**. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 341 (Pt 2): 233-249, 1999.
5. **Deyhimy DI, Fleming NW, Brodkin IG and Liu H**. Anesthetic preconditioning combined with postconditioning offers no additional benefit over preconditioning or postconditioning alone. *Anesth Analg* 105: 316-324, 2007.
6. **Ebel D., Toma O., Weber N.C., Huhn R., Preckel B. and Schlack W.** Hyperglycaemia blocks anaesthetic-induced preconditioning by desflurane during the mediator phase. *Eur J Anaesthesiol* 22 Supp.: A 165, 2005.
7. **Ebel D, Mullenheim J, Frassdorf J, Heinen A, Huhn R, Bohlen T, Ferrari J, Sudkamp H, Preckel B, Schlack W and Thamer V**. Effect of acute hyperglycaemia and diabetes mellitus with and without short-term insulin treatment on myocardial ischaemic late preconditioning in the rabbit heart in vivo. *Pflugers Arch* 446: 175-182, 2003.
8. **Feng J, Lucchinetti E, Ahuja P, Pasch T, Perriard JC and Zaugg M**. Isoflurane postconditioning prevents opening of the mitochondrial permeability transition pore through inhibition of glycogen synthase kinase 3beta. *Anesthesiology* 103: 987-995, 2005.

9. **Gateau-Roesch O, Argaud L and Ovize M**. Mitochondrial permeability transition pore and postconditioning. *Cardiovasc Res* 70: 264-273, 2006.
10. **Gross ER and Gross GJ**. Ligand triggers of classical preconditioning and postconditioning. *Cardiovasc Res* 70: 212-221, 2006.
11. **Hausenloy DJ, Maddock HL, Baxter GF and Yellon DM**. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res* 55: 534-543, 2002.
12. **Hausenloy DJ and Yellon DM**. Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc Res* 70: 240-253, 2006.
13. **Jelesoff NE, Feinglos M, Granger CB and Califf RM**. Outcomes of diabetic patients following acute myocardial infarction: a review of the major thrombolytic trials. *Coron Artery Dis* 7: 732-743, 1996.
14. **Kehl F, Krolikowski JG, Mraovic B, Pagel PS, Warltier DC and Kersten JR**. Hyperglycemia prevents isoflurane-induced preconditioning against myocardial infarction. *Anesthesiology* 96: 183-188, 2002.
15. **Kersten JR, Schmeling TJ, Orth KG, Pagel PS and Warltier DC**. Acute hyperglycemia abolishes ischemic preconditioning in vivo. *Am J Physiol* 275: H721-H725, 1998.
16. **Kersten JR, Toller WG, Gross ER, Pagel PS and Warltier DC**. Diabetes abolishes ischemic preconditioning: role of glucose, insulin, and osmolality. *Am J Physiol Heart Circ Physiol* 278: H1218-H1224, 2000.
17. **Krolikowski JG, Bienengraeber M, Weihrauch D, Warltier DC, Kersten JR and Pagel PS**. Inhibition of mitochondrial permeability transition enhances isoflurane-induced cardioprotection during early reperfusion: the role of mitochondrial KATP channels. *Anesth Analg* 101: 1590-1596, 2005.
18. **Kuusisto J, Mykkanen L, Pyorala K and Laakso M**. NIDDM and its metabolic control predict coronary heart disease in elderly subjects. *Diabetes* 43: 960-967, 1994.
19. **Mullenheim J, Frassdorf J, Preckel B, Thamer V and Schlack W**. Ketamine, but not S(+)-ketamine, blocks ischemic preconditioning in rabbit hearts in vivo. *Anesthesiology* 94: 630-636, 2001.
20. **Mullenheim J, Schlack W, Frassdorf J, Heinen A, Preckel B and Thamer V**. Additive protective effects of late and early ischaemic preconditioning are mediated by the opening of KATP channels in vivo. *Pflugers Arch* 442: 178-187, 2001.
21. **Obal D, Dettwiler S, Favoccia C, Scharbatke H, Preckel B and Schlack W**. The influence of mitochondrial KATP-channels in the cardioprotection of preconditioning and postconditioning by sevoflurane in the rat in vivo. *Anesth Analg* 101: 1252-1260, 2005.
22. **Obal D, Preckel B, Scharbatke H, Mullenheim J, Hoterkes F, Thamer V and Schlack W**. One MAC of sevoflurane provides protection against reperfusion injury in the rat heart in vivo. *Br J Anaesth* 87: 905-911, 2001.
23. **Preckel B, Schlack W, Comfere T, Obal D, Barthel H and Thamer V**. Effects of enflurane, isoflurane, sevoflurane and desflurane on reperfusion injury after regional myocardial ischaemia in the rabbit heart in vivo. *Br J Anaesth* 81: 905-912, 1998.
24. **Toma O, Weber NC, Wolter JI, Obal D, Preckel B and Schlack W**. Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart in vivo. *Anesthesiology* 101: 1372-1380, 2004.
25. **Tsang A, Hausenloy DJ, Mocanu MM and Yellon DM**. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res* 95: 230-232, 2004.
26. **Zhao ZQ and Vinten-Johansen J**. Postconditioning: reduction of reperfusion-induced injury. *Cardiovasc Res* 70: 200-211, 2006.

Chapter 9

Physiological levels of glutamine prevent morphine induced preconditioning in the isolated rat heart

André Heinen, Ragnar Huhn, Markus W. Hollmann, Benedikt Preckel, Coert J. Zuurbier, Wolfgang Schlack, Jan Fräβdorf, Nina C. Weber

Submitted for Publication

ABSTRACT

Morphine induces cardioprotection against ischaemia-reperfusion injury. While aiming to investigate the underlying signal transduction cascade of morphine preconditioning in isolated Langendorff-perfused rat hearts, the expected cardioprotection was not detectable. Thus, we investigated the influence of different preconditioning protocols and substrate conditions on cardioprotection in this experimental model. Isolated rat hearts underwent 35 min global ischaemia followed by 60 min reperfusion. Morphine PC was initiated by 3 cycles of 5 min 1 μM morphine with either 5 min washout [3PC5(5)] or 15 min washout [3PC5(15)] before ischaemia; by 15 min morphine with 15 min washout before ischaemia [PC15(15)]; or by 15 min 10 μM morphine with 15 min washout [PC15(15)-10μM]. Ischaemic preconditioning was initiated by 3 cycles of 3 min ischaemia; in another group, hearts received 1 μM morphine continuously for 10 min before ischaemia until the end of reperfusion [continued morphine]. To investigate the effects of glutamine, two groups received a glutamine-free perfusate: a control group, and a morphine preconditioning group [3PC5(15)]. Ischaemic preconditioning reduced infarct size by 75%, and continued morphine by 46% compared to control group. With the glutamine containing perfusate, none of the morphine PC pretreatments had an effect on infarct size. In glutamine-free perfusate, 3 cycles of 5 min 1 μM morphine with 15 min washout reduced infarct size from 45%±8% (control) to 20%±5% (3PC5(15). Cardioprotection by morphine induced preconditioning is model dependent: in the isolated rat heart, morphine preconditioning is prevented by a glutamine containing perfusate.

INTRODUCTION

Stimulation of opioid receptors both by endogenous and exogenous opioids increases the resistance of the myocardium against ischaemia and reperfusion injury. (4; 18; 20; 24)
The mechanisms by which opioids protect the myocardium share common pathways with ischaemic preconditioning. It is shown that opening of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels, which are involved in regulation of mitochondrial functions, is a key step to mediate both morphine and ischaemic preconditioning induced cardioprotection, possibly due to inhibition of mitochondrial permeability transition pore (mPTP) opening. (4; 15) In 2002, Hausenloy et al. demonstrated that prevention of mPTP opening is involved in ischaemic preconditioning. (6)
In this context, we initially aimed to investigate, whether morphine also induces preconditioning by prevention of mPTP opening in the isolated rat heart. However, the expected protective effect of morphine was surprisingly not detectable in our experimental model of the isolated Langendorff-perfused rat heart.
Based on these unexpected results, we hypothesized in the present study that morphine induced cardioprotection might be strongly dependent on the experimental conditions and the protocol by which morphine is administered. Most studies investigating the protective potency of morphine in intact hearts are conducted using non-classical preconditioning protocols (i.e. without washout of morphine before ischaemia), or in *in vivo* models where, dependent on the half-time of morphine, it can be assumed that morphine is still present during ischaemia. In addition, differences in experimental conditions related to the substrates present in the perfusate may also affect cardioprotective interventions. Recent work suggests e.g. that glutamine may have cardioprotective potential. (11)
Thus, we investigated whether the cardioprotective effect of morphine induced preconditioning in the isolated rat heart depends on the preconditioning protocol and experimental substrate conditions.

MATERIALS AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and approved by the Animal Ethical Committee of the University of Amsterdam.

Chemicals and reagents

Morphine-HCl was purchased from Centrafarm (Etten-Leur, The Netherlands). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Surgical preparation

Sixty-one male, Wistar rats (Charles River, Netherlands) weighing 250–350 g were used for these studies. Rats were maintained on a 12:12 light/dark schedule (lights on at 0600 hours) with food and water provided *ad libitum*. The rats were acclimated to the local animal facility for at least 7 days prior to use in an experiment. Rats were anesthetized with pentobarbital (90 mg/kg i.p.). After thoracotomy, the aorta was cannulated in situ and perfusion of the myocardium with Krebs-Henseleit solution was started before excision of the heart to reduce ischaemic periods. Then, hearts were mounted on a Langendorff system and were perfused at constant pressure (80 mmHg) with Krebs-Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.5 EDTA, 2.25 $CaCl_2$, 11 glucose, 0.5 glutamine, 1 lactate, and 0.1 pyruvate at 37°C. A fluid filled balloon was inserted into the left ventricle and end-diastolic pressure was set at 1-4 mmHg. All hearts underwent a stabilization period of 20 minutes. Heart rate, myocardial function (isovolumetric left ventricular pressure), coronary flow, left ventricular end-diastolic pressure, and dP/dt_{max} were measured continuously. Arrhythmic intervals were not used for data analysis. The rate pressure product was calculated as heart rate * (maximal left ventricular pressure – left ventricular end-diastolic pressure).

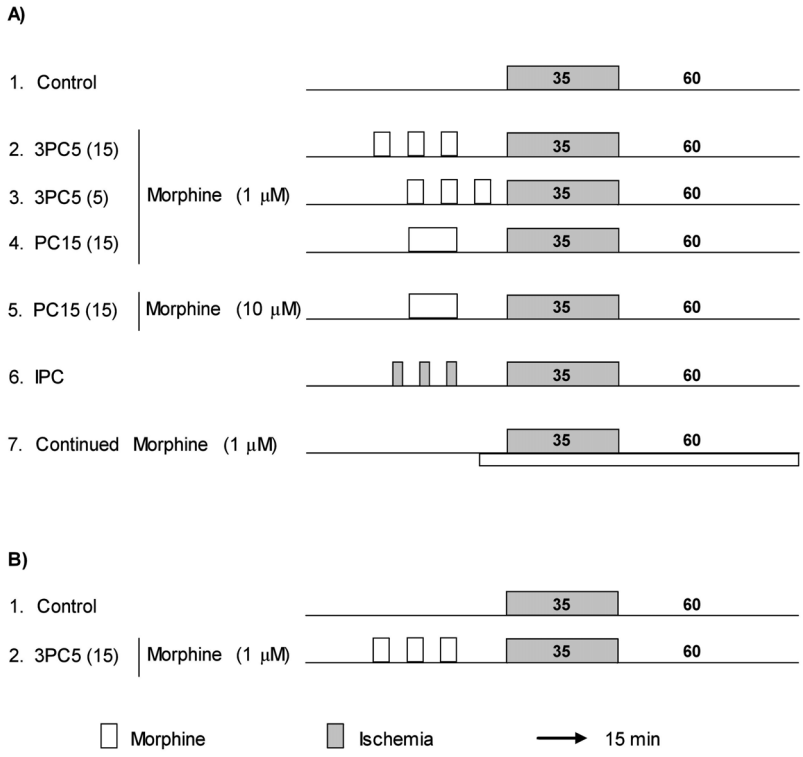
Experimental design

To investigate whether morphine induces preconditioning in the isolated rat heart, we conducted a first series (series 1) of experiments (Fig. 1, panel A). Hearts were assigned to one of seven experimental groups. Hearts of all groups underwent 35 minutes of ischaemia followed by 60 minutes of reperfusion. In control group, hearts were kept under baseline conditions for 40 minutes prior to ischaemia. To investigate whether morphine induces preconditioning, 1 μM morphine-HCl was given in three different preconditioning protocols: 3 cycles of 5 minutes, interspersed by 5 minutes washout 15 min prior to ischaemia (3PC5 (15), group 2), 3 cycles of 5 minutes, interspersed by 5 minutes washout 5 min prior to ischaemia (3PC5 (5), group 3), and for 15 minutes 15 minutes prior to ischaemia (PC15 (15), group 4). To test, whether a high concentration of morphine induces preconditioning, group 5 received 10 μM morphine for 15 minutes 15 minutes prior to ischaemia (PC15 (15) Morphine 10 μM). As positive controls, ischaemic preconditioning

group (group 6) underwent three cycles of 3 minutes ischaemia 15 minutes prior to ischaemia, and group 7 received 1 µM morphine for 10 minutes before ischaemia and throughout reperfusion (continued morphine). Morphine was dissolved in NaCl (0.9%) and separately infused into a mixing chamber placed in the perfusion system.

At 60 min of reperfusion, hearts were frozen at -20°C. Subsequently, infarct sizes were determined by triphenyl-tetrazolium chloride (TTC) staining. Therefore, heart slices (7-9 per heart) were incubated with 0.75% TTC solution for 10 min at 37°C and fixed in 4% formalin solution for 24 h at room temperature. The infarcted area was determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) by two researchers in a blinded manner.

Figure 1:



Experimental protocol. *Panel A:* Experimental series 1. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1 mM), pyruvate (0.1 mM) and glutamine (0.5 mM) as substrates. *Panel B:* Experimental series 2. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1 mM) and pyruvate (0.1 mM) as substrates.

Based on our results from experimental series 1, we conducted subsequently a second series of experiments where we investigated the impact of glutamine on morphine induced preconditioning. For this, we conducted experiments under the same conditions as in series 1 except that we perfused the hearts with glutamine-free Krebs-Henseleit solution. The experimental protocol (Fig. 1, panel B) corresponded to the first two groups of series 1, e.g. a control group and a 3PC5 (15) group.
Infarct sizes were determined as described above.

Statistical analysis
Data are presented as mean ± S.D. Group differences were analyzed (SPSS Science Software, version 12.0.1) with use of ANOVA followed by Dunnet's post hoc test for experimental series 1, and with the Student *t* test for experimental series 2. Changes were considered statistically significant when the *P* value was less than 0.05.

RESULTS

Experimental series 1:
No differences in body or heart weight were observed between the groups (Table 1 panel A).

Infarct size

Infarct size was 26% ± 6% (n = 8) in controls and was neither affected by any of the three preconditioning protocols with 1 μM morphine (3PC5 (15): 33% ± 7% (n = 7); 3PC5 (5): 25% ± 10% (n = 7); PC15 (15): 25% ± 5% (n = 6), all n.s. vs. control), nor by the administration of 10 μM morphine (3PC5 (15): 26% ± 8% (n = 7), n.s. vs. control). (Figure 2, panel A)
Ischaemic preconditioning by three cycles of 3 minutes ischaemia reduced infarct size to 6% ± 3% (n = 7, $P < 0.05$ vs. control). Continuous administration of morphine reduced infarct size to 14% ± 7% (n = 7, $P < 0.05$ vs. control). (Figure 2, panel A)

Tabel 1:

A) with glutamine

	Body weight (g)	Heart weight wet (g)	Heart weight dry (mg)	Time of max. ischaemic contracture (min)	Level of max. ischaemic contracture (mmHg)
Control	325±17	1.5±0.2	176±18	17±1	81±14
3PC5(15)	332±27	1.5±0.2	183±16	17±1	86±6
3PC5(5)	340±37	1.6±0.2	177±7	17±2	92±2
PC15(15)	338±22	1.5±0.1	185±12	17±0	94±8
PC15(15)-10μM	322±18	1.5±0.2	185±7	17±2	77±13
IPC	330±35	1.5±0.1	182±17	17±2	74±8
continued Morphine	334±27	1.5±0.1	185±13	17±2	87±16

B) without glutamine

	Body weight (g)	Heart weight wet (g)	Heart weight dry (mg)	Time of max. ischaemic contracture (min)	Level of max. ischaemic contracture (mmHg)
Control	320±23	1.4±0.1	172±6	17±1	82±11
3PC5(15)	316±13	1.5±0.2	180±13	17±2	68±8 [a]

Data are mean±S.D.; [a] $P<0.05$ vs. control. PC = preconditioning; IPC = ischaemic preconditioning

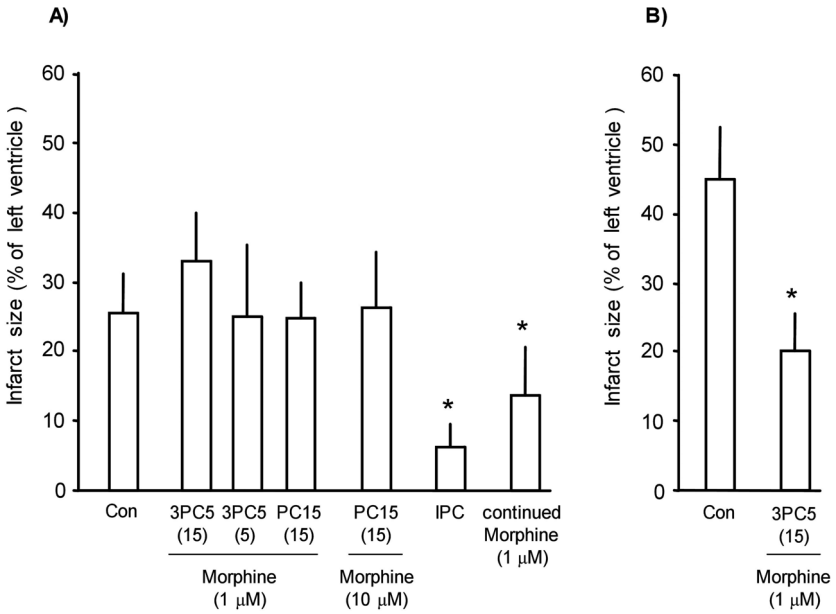
Haemodynamics

No significant differences in rate pressure product, left ventricular end-diastolic pressure, and dP/dt_{max} were observed between the experimental groups during baseline conditions, and at the beginning of ischaemia. (Fig. 3) During the three cycles of ischaemic preconditioning, we observed a statistically lower rate pressure product and dP/dt_{max}. The latter remained reduced at 5 minutes of each reperfusion period of the preconditioning cycles. During reperfusion after the prolonged period of ischaemia, the left ventricular end-diastolic pressure was lower in the ischaemic preconditioning group compared with controls, and at the end of the experiment, rate pressure product, dP/dtmax, and phasic left ventricular pressure was higher in the ischaemic preconditioning group. There was no

difference in heart rate compared with controls at baseline and during reperfusion (Table 2).

There was no significant difference between all groups regarding the time when left ventricular contracture reached the maximum, and the level of maximal left ventricular ischaemic contracture (Table 1, panel A)

Figure 2:



Panel A shows infarct sizes as a percentage of the left ventricle in experimental series 1; *Panel B* shows infarct sizes as a percentage of the left ventricle in experimental series 2. Data are presented as mean±S.D.; **P* < 0.05 vs. control.

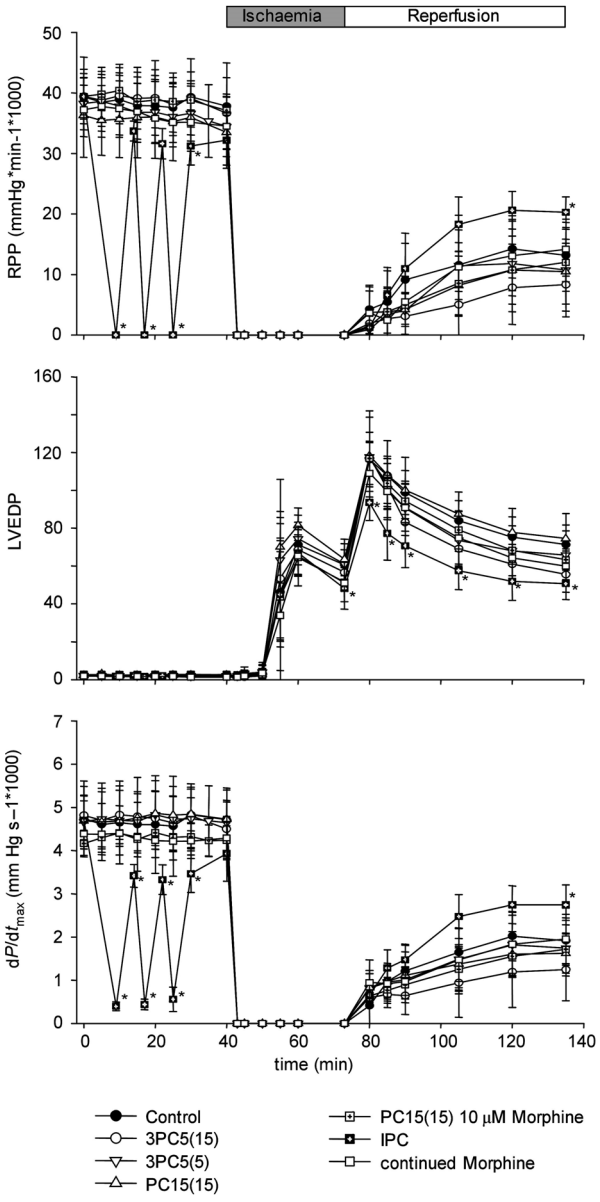
Experimental series 2:

No differences in body or heart weight were observed between the groups (Table 1, panel B).

Infarct size

Infarct size was 45% ± 8% (n = 6) in controls. (Figure 2, panel B) Preconditioning by administration of 1 μM morphine (3PC5 (15)) reduced infarct size to 20% ± 5% (n = 6, P < 0.05 vs. control).

Figure 3:



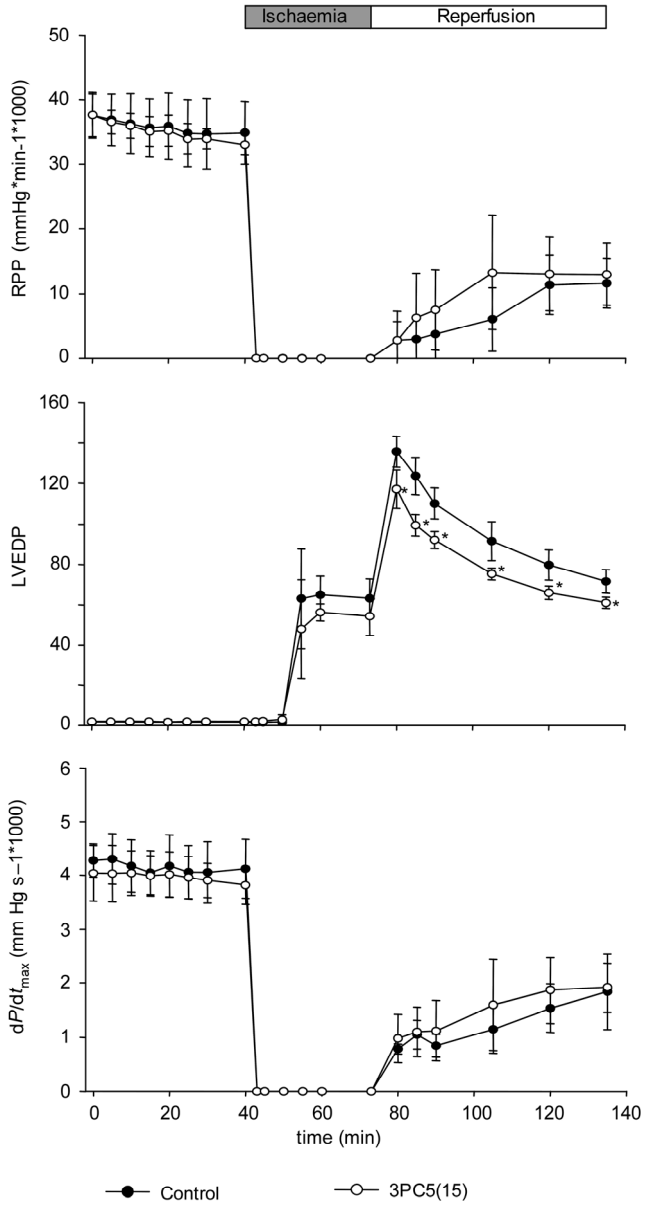
Line plots showing the time course of rate pressure product (RPP), left ventricular end-diastolic pressure (LVEDP), and dP/dt_{max} during experimental series 1. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1 mM), pyruvate (0.1 mM) and glutamine (0.5 mM) as substrate. Data are presented as mean±S.D.; * $P < 0.05$ vs. control.

Table 2:

	Baseline	Reperfusion (min)			
		5	15	30	60
Heart rate (bpm)					
Con	303 ± 52	160 ± 132	253 ± 84	268 ± 45	252 ± 78
3PC5(15)	300 ± 19	132 ± 141	155 ± 124	156 ± 146	246 ± 102
3PC5(5)	291 ± 19	132 ± 118	156 ± 113	248 ± 116	218 ± 102
PC15(15)	285 ± 39	106 ± 91	105 ± 115	188 ± 109	233 ± 94
PC15(15)-10μM	297 ± 38	176 ± 124	210 ± 112	265 ± 44	257 ± 45
IPC	318 ± 36	191 ± 89	247 ± 71	243 ± 51	279 ± 44
continued Morphine	292 ± 20	117 ± 140	235 ± 111	270 ± 48	266 ± 45
Phasic LVP (mmHg)					
Con	130.1 ± 10.1	28.2 ± 13.9	32.4 ± 18.3	44.3 ± 22.3	50.5 ± 17.5
3PC5(15)	131.0 ± 8.5	12.8 ± 11.7	13.3 ± 15.7	22.0 ± 22.3	31.3 ± 17.8
3PC5(5)	132.3 ± 20.9	22.8 ± 16.5	22.9 ± 19.1	40.0 ± 24.0	44.5 ± 19.3
PC15(15)	126.6 ± 8.9	26.5 ± 15.0	26.9 ± 17.3	37.5 ± 15.5	42.4 ± 11.0
PC15(15)-10μM	133.7 ± 11.1	15.6 ± 14.2	19.5 ± 16.0	32.9 ± 21.5 [a]	47.9 ± 15.3 [a]
IPC	125.5 ± 9.3	35.5 ± 15.2	48.4 ± 20.3	77.0 ± 17.8	73.9 ± 11.9
continued Morphine	127.6 ± 9.1	15.1 ± 10.7	22.0 ± 12.4	44.0 ± 21.5	53.2 ± 13.7
CF (ml min^{-1})					
Con	12.5 ± 2.5	7.6 ± 1.7	7.8 ± 1.8	7.9 ± 1.8	7.7 ± 1.9
3PC5(15)	14.3 ± 0.8	8.4 ± 1.1	8.9 ± 1.5	9.1 ± 1.9	8.9 ± 2.0
3PC5(5)	13.8 ± 2.6	8.9 ± 1.5	8.5 ± 1.4	8.5 ± 1.4	8.5 ± 1.5
PC15(15)	12.3 ± 2.3	8.7 ± 2.4	8.7 ± 2.4	8.1 ± 1.1	7.9 ± 0.9
PC15(15)-10μM	13.5 ± 2.0	8.1 ± 0.8	8.1 ± 0.8	8.2 ± 1.0	8.1 ± 1.6
IPC	12.7 ± 1.3	10.6 ± 1.3 [a]	10.2 ± 1.1	10.0 ± 1.4	9.4 ± 1.8
continued Morphine	13.4 ± 1.3	9.8 ± 1.5	9.8 ± 1.9	9.7 ± 2.1	9.4 ± 2.4

Data are mean±S.D.; [a] $P<0.05$ vs Con. Phasic LVP = systolic left ventricular pressure - enddiastolic left ventricular pressure; CF = coronary flow; Con = control; PC = preconditioning; IPC = ischaemic preconditioning.

Figure 4:



Line plots showing the time course of rate pressure product (RPP), left ventricular end-diastolic pressure (LVEDP), and dP/dt_{max} during experimental series 2. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1 mM) and pyruvate (0.1 mM) as substrate. Data are presented as mean±S.D.; * $P < 0.05$ vs. control.

Haemodynamics

No significant differences in RPP, LVEDP and dP/dt_{max} were observed between the experimental groups during baseline conditions, and at the beginning of ischaemia. (Figure 4) During reperfusion after the prolonged period of ischaemia, the LVEDP was lower in the 3PC5 (15) group compared with controls. There was no difference in HR, phasic LVP and CF compared with controls at baseline and during reperfusion (Table 3).

There was no significant difference between both groups regarding the time when left ventricular contracture reached the maximum. (Table 1, panel B) The level of maximal left ventricular ischaemic contracture was significantly reduced in 3PC5(15) (68 ± 8 mm Hg vs. 82 ± 11 mmHg, $P < 0.05$)

Table 3:

	Baseline	Reperfusion (min)			
		5	15	30	60
Heart rate (bpm)					
Con	315 ± 27	144 ± 150	202 ± 151	198 ± 123	291 ± 33
3PC5(15)	316 ± 25	84 ± 119	249 ± 51	306 ± 28	259 ± 59
Phasic LVP (mmHg)					
Con	119.8 ± 13.7	14.5 ± 8.6	15.1 ± 9.4	24.9 ± 16.0	40.5 ± 13.8
3PC5(15)	119.2 ± 10.6	17.2 ± 16.4	28.4 ± 18.5	42.3 ± 25.2	50.3 ± 13.0
CF (ml min^{-1})					
Con	14.2 ± 1.4	7.5 ± 1.1	7.9 ± 1.1	7.9 ± 1.0	7.7 ± 0.9
3PC5(15)	14.6 ± 1.5	8.5 ± 1.8	8.9 ± 1.6	9.0 ± 1.5	8.9 ± 1.7

Data are mean±S.D.; Phasic LVP = systolic left ventricular pressure - enddiastolic left ventricular pressure; CF = coronary flow; Con = control; PC = preconditioning

DISCUSSION

The main findings of our study are that *a)* in the isolated Langendorff-perfused rat heart morphine administration in a preconditioning manner does not induce protection of the

myocardium in the presence of physiological concentrations of glutamine, while both continued administration of morphine, and ischaemic preconditioning protect the myocardium, and that *b)* morphine administration in a preconditioning manner induces myocardial protection in the absence of glutamine.

It is well known that stimulation of opioid receptors by both endogenous and exogenous opioids enhances the resistance of the myocardium against ischaemia-reperfusion injury. (16; 17; 24; 25) Schultz et al. showed that the nonselective opioid receptor antagonist naloxone abrogated the cardioprotective effect of ischaemic preconditioning, (20) and, furthermore, that exogenous opioid receptor stimulation by morphine initiates cardioprotection. (18) In a later study, Schultz et al. demonstrated that the cardioprotective effect of ischaemic preconditioning was mediated by δ_1-opiod receptor activation. Pharmacological blockade of neither μ-receptors nor k-opioid receptors had an effect on ischaemic preconditiong. (19) There is evidence that also morphine-induced preconditioning is mediated via activation of δ-opiod receptors. (21)

Furthermore, it was shown that the regulation of mitochondrial function by activation of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels plays a central role in morphine induced cardioprotection,. (4; 14) Ludwig el al. demonstrated that morphine enhanced isoflurane induced preconditioning via activation of mK_{ATP} channels. (12) The involvement of mitochondria in morphine induced cardioprotection is supported by data from our group, showing that morphine causes a translocation of hexokinase to the mitochondria. (26) The interaction of hexokinase with the mitochondria may inhibit apoptosis through inhibition of mPTP opening. (13) Prevention of mPTP opening due to alterations in mitochondrial function is involved in ischemic preconditioning. (6) Thus, we initially aimed at investigating the role of mPTP in morphine induced preconditioning in the isolated Langendorff-perfused rat heart. However, we failed to detect a protective effect of morphine. Based on this surprising finding we investigated whether morphine induced preconditioning depends on the preconditioning protocol. Our results show that morphine does not initiate cardioprotection when administered in a preconditioning manner i.e. with a washout phase where the substance is no longer present during ischaemia and reperfusion. In contrast, with the continuous administration of morphine, cardioprotection could be observed and infarct size was reduced. Many of the studies investigating the cardioprotective effects of morphine were conducted using either *in vivo* models of myocardial infarction or the Langendorff perfusion model. In the case of *in vivo* models, when the drug is given prior to ischaemia, it is difficult to discriminate between pharmacological actions that occur before ischaemia, i.e. preconditioning, or during

ischaemia and reperfusion because the substance will still be present in the tissues. Dependent on the half-time of morphine, it can be assumed that morphine is still present during ischaemia.

In most prior studies investigating the protective effect of morphine on ischaemia-reperfusion injury in the isolated heart model, morphine was administered until the onset of ischaemia and/or throughout the reperfusion period, i.e., also not in a classical preconditioning protocol. Cohen et al. demonstrated in the isolated rabbit heart that 5 minutes of perfusion with 0.3 μM morphine initiates preconditioning. (4) In their study, morphine administration was followed by 15 minutes of perfusion with morphine-free perfusate to allow a washout of the drug before the global ischemia. (4) In contrast to their study, we did not detect an infarct size reducing effect of morphine in a similar protocol, i.e. when morphine treatment was not given until the onset of the global ischemia. Therfore, it is unlikely that the contradictory findings of both studies are caused by different experimental protocols. Furthermore, the morphine concentration of 1 μM that was used in the present study has been shown to induce the strongest preconditioning effect in ventricular myocytes. (10) Interestingly, ongoing experiments from a collaborating laboratory (Department of Anaesthesiology, University of Düsseldorf, Germany) investigating a different effect of morphine on the isolated rat heart showed a strong preconditioning effect of 1 μM morphine in isolated Langendorff-perfused rat hearts (preliminary data).

Therefore, we hypothesized that the protective properties of morphine are not only dependent on the administration protocol, but also affected by the experimental conditions.

Recently, there is increasing interest in the question, why the translation of beneficial preconditioning effects obtained in animal models into the clinical setting has been disappointing.(for review see (5; 23) One of the likely factors relates to that laboratory conditions often deviates largely from the normal physiological conditions. For example, the substrates and concentrations thereof used in isolated heart perfusion studies often deviates from that found in vivo. (2) Furthermore, discrepancies in the results of animal studies can partially be caused by the failure to use standardized models and research protocols. (1)

A comparison of the exact models and research protocols of our study and the study from the collaborating laboratory showed a difference in the substrate composition of the perfusates. In contrast to the study conducted at the University of Düsseldorf, where only glucose and pyruvate were present, the perfusate used in the first series of our experiments contained a mixture of glucose and pyruvate, lactate, and *glutamine* at physiological

concentrations to simulate physiological substrate conditions. (2; 3; 22) As stated above, this metabolic profile was specifically chosen to minimize as much as possible problems associated with the translation from the laboratory condition to the in vivo condition. It is already known that glutamine at higher than physiological concentrations (1.25 – 2.5mM) protect the heart against I/R injury. (8; 9; 11) Our present study not only shows that glutamine already at physiological concentrations protects the heart against I/R injury, but that this concentration of glutamine also prevents morphine-induced preconditioning.

A limitation of this study is that it cannot provide deeper insight into the exact mechanism by which glutamine and morphine induced preconditioning interfere. It has been demonstrated by Liu et al. that the cardioprotective effect of glutamine is mediated via activation of the hexosamine biosynthesis pathway and increased protein O-linked N-acetylglucosamine (O-GlcNAc) levels. (11) Recently, Jones et al. demonstrated that "O-GlcNAc signaling represents a unique endogenously recruitable mechanism of cardioprotection that may involve direct modification of mitochondrial proteins critical for survival such as voltage-dependent anion channel." (7) It is possible that at least parts of this pathway are also involved in the signal transduction of morphine-induced preconditioning. Future studies are needed to clarify this possible interaction.

Taken together, this study demonstrates that morphine can induce preconditioning, but that the protective effect strongly depends on experimental conditions, e.g. both the administration protocol and the substrate conditions. The data from our study might suggest that a preconditioning effect of morphine may be non-existent due to the physiological presence of glutamine. On the other hand, from a continuous presence of morphine during ischemia and reperfusion a cardioprotective effect of morphine could still be expected also in the absence of a preconditioning effect. Any translation from our highly artificial model to the in vivo situation should be done with caution. Therefore, we hope that the results of this study may contribute to a more critical view on experimental conditions and experimental settings when translating conclusions from laboratory studies to the in vivo condition.

REFERENCES

1. **Bolli R, Becker L, Gross G, Mentzer R, Jr., Balshaw D and Lathrop DA**. Myocardial protection at a crossroads: the need for translation into clinical therapy. *Circ Res* 95: 125-134, 2004.
2. **Chatham JC, Des RC and Forder JR**. Evidence of separate pathways for lactate uptake and release by the perfused rat heart. *Am J Physiol Endocrinol Metab* 281: E794-E802, 2001.

3. **Chatham JC, Gao ZP, Bonen A and Forder JR**. Preferential inhibition of lactate oxidation relative to glucose oxidation in the rat heart following diabetes. *Cardiovasc Res* 43: 96-106, 1999.
4. **Cohen MV, Yang XM, Liu GS, Heusch G and Downey JM**. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K_{ATP} channels. *Circ Res* 89: 273-278, 2001.
5. **Dirksen MT, Laarman GJ, Simoons ML and Duncker DJ**. Reperfusion injury in humans: a review of clinical trials on reperfusion injury inhibitory strategies. *Cardiovasc Res* 74: 343-355, 2007.
6. **Hausenloy DJ, Maddock HL, Baxter GF and Yellon DM**. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res* 55: 534-543, 2002.
7. **Jones SP, Zachara NE, Ngoh GA, Hill BG, Teshima Y, Bhatnagar A, Hart GW and Marban E**. Cardioprotection by N-acetylglucosamine linkage to cellular proteins. *Circulation* 117: 1172-1182, 2008.
8. **Khogali SE, Harper AA, Lyall JA and Rennie MJ**. Effects of L-glutamine on post-ischaemic cardiac function: protection and rescue. *J Mol Cell Cardiol* 30: 819-827, 1998.
9. **Khogali SE, Pringle SD, Weryk BV and Rennie MJ**. Is glutamine beneficial in ischemic heart disease? *Nutrition* 18: 123-126, 2002.
10. **Liang BT and Gross GJ**. Direct preconditioning of cardiac myocytes via opioid receptors and K_{ATP} channels. *Circ Res* 84: 1396-1400, 1999.
11. **Liu J, Marchase RB and Chatham JC**. Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein O-GlcNAc levels. *J Mol Cell Cardiol* 42: 177-185, 2007.
12. **Ludwig LM, Patel HH, Gross GJ, Kersten JR, Pagel PS and Warltier DC**. Morphine enhances pharmacological preconditioning by isoflurane: role of mitochondrial K_{ATP} channels and opioid receptors. *Anesthesiology* 98: 705-711, 2003.
13. **Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K, Chandel NS, Thompson CB, Robey RB and Hay N**. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell* 16: 819-830, 2004.
14. **McPherson BC and Yao Z**. Signal transduction of opioid-induced cardioprotection in ischemia-reperfusion. *Anesthesiology* 94: 1082-1088, 2001.
15. **Murphy E and Steenbergen C**. Preconditioning: The mitochondrial connection. *Annu Rev Physiol* 69: 51-67, 2007.
16. **Peart JN, Gross ER and Gross GJ**. Opioid-induced preconditioning: recent advances and future perspectives. *Vascul Pharmacol* 42: 211-218, 2005.
17. **Peart JN and Gross GJ**. Exogenous activation of delta- and kappa-opioid receptors affords cardioprotection in isolated murine heart. *Basic Res Cardiol* 99: 29-37, 2004.
18. **Schultz JE, Hsu AK and Gross GJ**. Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. *Circ Res* 78: 1100-1104, 1996.
19. **Schultz JE, Hsu AK and Gross GJ**. Ischemic preconditioning in the intact rat heart is mediated by delta1- but not mu- or kappa-opioid receptors. *Circulation* 97: 1282-1289, 1998.
20. **Schultz JE, Rose E, Yao Z and Gross GJ**. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. *Am J Physiol* 268: H2157-H2161, 1995.
21. **Schultz JJ, Hsu AK and Gross GJ**. Ischemic preconditioning and morphine-induced cardioprotection involve the delta (delta)-opioid receptor in the intact rat heart. *J Mol Cell Cardiol* 29: 2187-2195, 1997.

22. **Stein WH and Moore S**. The free amino acids of human blood plasma. *J Biol Chem* 211: 915-926, 1954.
23. **Yellon DM and Hausenloy DJ**. Myocardial reperfusion injury. *N Engl J Med* 357: 1121-1135, 2007.
24. **Zhang Y, Irwin MG and Wong TM**. Remifentanil preconditioning protects against ischemic injury in the intact rat heart. *Anesthesiology* 101: 918-923, 2004.
25. **Zhang Y, Irwin MG, Wong TM, Chen M and Cao CM**. Remifentanil preconditioning confers cardioprotection via cardiac kappa- and delta-opioid receptors. *Anesthesiology* 102: 371-378, 2005.
26. **Zuurbier CJ, Eerbeek O and Meijer AJ**. Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution. *Am J Physiol Heart Circ Physiol* 289: H496-H499, 2005.

Chapter 10

Main Conclusions and General Discussion

MAIN CONCLUSIONS

In this thesis, we investigated mechanisms of cardioprotection by preconditioning and postconditioning. In this context, we focused on the involvement of mitochondria and the mechanisms, by which mitochondrial function is regulated in cardioprotective interventions.

Mitochondrial bioenergetics can be regulated by activation of mK_{Ca} channels
In the trigger mechanism of preconditioning are both the activation of mitochondrial potassium channels and the generation of reactive oxygen species involved. We investigated the effects of mK_{Ca} channel activation on mitochondrial bioenergetics. **(chapter 2, 3)** The mK_{Ca} channel activator NS1619 increased mitochondrial respiration in a dose-dependent manner. We could show that mitochondrial reactive oxygen species are increased by low concentrations of NS1619 lacking effect on mitochondrial membrane potential. We therefore conclude that mitochondria are capable to generate reactive oxygen species in response to mK_{Ca} channel activation.

The effect of mK_{Ca} channel activation on mitochondrial respiration is age-dependent
Aging is associated with multiple alterations and dysfunctions in cellular processes. In **chapter 2 and 3** we demonstrated that mK_{Ca} channel activation stimulated mitochondrial respiration, an effect that might be age-dependent. We showed in isolated cardiac mitochondria that the effect of the mK_{Ca} channel activator NS1619 on mitochondrial respiration is lost with increasing age **(chapter 4)**. The diminished effects of mK_{Ca} opening on mitochondrial respiration might be one underlying mechanism of the decreased protective potency of preconditioning in the aged myocardium.

The noble gas helium induces preconditioning via activation of mK_{Ca} channels
Cardiac preconditioning can be initiated by the noble gas helium. We demonstrated that the infarct size-reducing effect of helium-induced preconditioning is abrogated by administration of the mK_{Ca} channel antagonist iberotoxin **(chapter 5)**. Furthermore, we demonstrated that helium causes a mild uncoupling of mitochondrial respiration, an effect that was also blocked by pre-treatment with iberotoxin. Therefore, we conclude that helium causes mitochondrial uncoupling, and might induce preconditioning in young rats via mK_{Ca} channel activation.

Helium-induced preconditioning is abolished in both aged and pre-diabetic rats

Activation of mK_{Ca} channels is critically involved in helium-induced preconditioning **(chapter 5)**. In **chapter 4** we could show that the effect of mK_{Ca} channel activation on mitochondrial respiration is age-dependent. Based on these findings, we investigated the protective potency of helium in the aged myocardium. In this study, helium had no effect on infarct size and did not affect mitochondrial respiration in aged rat hearts **(chapter 5)**. Furthermore, we demonstrated in a similar study that helium had no effect on infarct size and mitochondrial respiration in hearts from pre-diabetic rats **(chapter 6)**. We suggest that the blockade of the protective effect of helium in aged and pre-diabetic rats could be associated with some defects at the level of the mK_{Ca} channel or its upstream signaling cascade.

Helium-induced late preconditioning is not associated with alterations in mitochondrial function during the mediator phase

In **chapter 7** we demonstrate that helium is able to induce late preconditioning in the rat heart. As shown in **chapter 5 and 6** of this thesis, helium causes a mild uncoupling of mitochondrial respiration shortly after helium administration. This mitochondrial effect of helium is not detectable during the mediator phase of late preconditiong, i.e. 6, 10, or 24 hours after the helium treatment. Therefore, we conclude that regulation of mitochondrial bioenergetics is not involved in the mediator signaling steps of helium-induced late preconditioning.

Hyperglycemia abolishes sevoflurane-induced postconditioning

It is hypothesized that hyperglycaemia might cause a loss of cardioprotective mechanisms. In **chapter 8**, we demonstrated that hyperglycaemia blocks cardioprotection by sevoflurane-induced postconditioning, and that this loss of cardioprotection can be restored by administration of the mPTP inhibitor Cyclosporin A.

Glutamine prevents morphine-induced preconditioning

In **chapter 9**, we initially aimed to investigate the impact of mPTP prevention in morphine-induced preconditioning, but we failed to detect a cardioprotective effect of morphine. Our results demonstrate that glutamine, which was present in the perfusion buffer to simulate physiological substrate conditions, prevents morphine-induced preconditioning.

GENERAL DISCUSSION

The general aim of this thesis was to investigate the role of cardiac mitochondria in the signaling pathways of cardioprotection. We focused on a) mechanisms by which mK_{Ca} channel activation regulates mitochondrial function, b) alterations in mitochondrial function caused by helium-induced preconditioning, and c) the impact of mPTP inhibition in cardioprotective interventions.

Since Murry et al. (16) first described the phenomenon of ischemic preconditioning many efforts were made to investigate the exact underlying mechanisms. Now, it is well accepted that activation of K^+ channels in the inner mitochondrial matrix is a key step during the trigger phase of preconditioning. (17) The exact mechanism by which K^+ influx into the mitochondrial matrix regulates mitochondrial function to initiate preconditioning is incompletely understood. Preconditioning can be blocked by the administration of free radical scavengers showing that small amounts of reactive oxygen species are required as signaling molecules in the signaling pathways of preconditioning. (14; 15; 19; 23) It is proposed that the reactive oxygen species are generated from the mitochondrial electron transport chain as consequence of regulations in mitochondrial bioenergetics due to K^+ channel activation. (6) However, the mechanism by which K^+ channel activation regulates mitochondrial function to cause reactive oxygen species is incompletely understood. In this thesis, we investigated whether activation of mK_{Ca} channels is capable to affect mitochondrial function to stimulate mitochondrial reactive oxygen species production. We could demonstrate that mK_{Ca} channel activation stimulate mitochondrial respiration. This finding is in line with a study from Sato et al., (21) who demonstrated that activation of mK_{Ca} channels increases flavoprotein oxidation in a dose-dependent manner in ventricular myocytes. Furthermore, we offer a possibility by which mK_{Ca} channel activation causes generation of reactive oxygen species by sub-maximal K^+ induced H^+ leak into the mitochondrial matrix. This slight increase in reactive oxygen species generation during the trigger phase of preconditioning might be one mechanism by which mK_{Ca} channel activation initiates preconditioning. However, it is still unclear whether K^+ channel-induced alterations in mitochondrial function are characteristic of all preconditioning stimuli to trigger cardioprotection, or whether differences in the signaling mechanisms exist.

Very recently, it has been shown that cardiac preconditioning can be initiated by administration of the noble gases helium, argon, and neon. (18) Pagel et al. demonstrated that these noble gases were capable to induce cardioprotection by activating prosurvival signaling kinases and inhibiting mPTP opening in rabbits *in vivo*. We confirm the

cardioprotective properties of helium demonstrating that helium induces preconditioning in the rat heart *in vivo*. In our study, the mK_{Ca} channel antagonist iberotoxin abrogated not only the infarct size-reducing effect of helium, but also alterations in mitochondrial respiration induced by helium. Therefore, we suggest that helium acts at least partially by mitochondrial K_{Ca} channel activation-induced alterations in mitochondrial respiration. Furthermore, we demonstrate that helium-induced preconditioning is blocked in both aged and pre-diabetic rats. It has been demonstrated that the cardioprotective potency of preconditioning is abolished or reduced in the diseased or senescent myocardium. (1-3; 5; 10; 11; 24; 25) There is evidence that the loss of cardioprotection in both the diabetic and the senescent heart is caused by defects in the signaling cascade at the level of the mitochondrion. (7; 13) Interestingly, the helium-induced alterations in mitochondrial respiration were also blocked in mitochondria isolated from aged and pre-diabetic rats. This finding further supports the impact of mK_{Ca} channel activation in helium-induced preconditioning, and, furthermore, it supports the possible role of mitochondria in the disease and age related loss of cardioprotection.

Mitochondria are not only involved in the trigger mechanisms of preconditioning, but also in possible end-effector mechanisms of cardioprotective interventions during ischemia and reperfusion. (8) A possible candidate as end-effector of cardioprotection is the mPTP. (9; 22) Inhibition of the mPTP by cyclosporine A confers cardioprotection. In the study described in chapter 8 of this thesis, we demonstrate that sevoflurane-induced postconditioning is blocked in hyperglycemic rats. Hyperglycemia has been shown to prevent cardioprotection by preconditioning. (12; 22) Furthermore, we demonstrate that inhibition of the mPTP is capable to restore the cardioprotective effect of sevoflurane-induced postconditioning in hyperglycemic rats. This finding emphasizes that mitochondria are potential targets for pharmacological therapy in the setting of myocardial ischemia and reperfusion.

Taken together, the results of this thesis strengthen and expand the knowledge that mitochondria are critically involved in multiple mechanistic steps of preconditioning and postconditioning. However, there are still multiple unanswered questions with regard to the role of mitochondria in cardioprotection, e.g. the upstream and downstream signaling steps of preconditioning are incompletely understood. The literature on preconditioning suggests many exclusive or crucially important signaling steps. This might be caused by the fact that in most studies mechanistic aspects rather than complete signaling networks are analyzed. This consideration should be taken into account when experimental results are interpreted and exclusive conclusions are drawn.

Limitations of the Experimental Models

The results obtained in this thesis have to be evaluated within the limitations of the experimental models used.

First, the phenomenon of ischemic preconditioning shows, that short periods of ischemia are capable of altering physiological processes. It is possible that isolated mitochondria or organs became ischemic during the isolation process, which in turn might influence experimental results.

Second, it has been demonstrated that cardiac mitochondria exist in two functionally distinct populations, as subsarcolemmal and interfibrillar mitochondria. (20) Differences between these groups exist among others with regard to calcium handling and susceptibility to ischemic damage. (4) We investigated a mixed population of both subsarcolemmal and interfibrillar mitochondria, and did not test for differences between mitochondrial subpopulations.

Third, the environment of isolated organs or organelles is highly artificial. The study described in chapter 9 demonstrates that e.g. substrate compositions can strongly influence isolated heart studies.

Therefore, a broad experimental approach that includes in vivo experiments as well as investigations at the organ, cell, and sub-cellular level can minimize the risk of drawing incorrect conclusions from experimental studies.

Clinical Implications

There is strong evidence that the resistance of the myocardium against ischemia and reperfusion can be increased by preconditioning and postconditioning in humans. Patients suffering from ischemic heart disease could possibly benefit from therapeutical interventions that induce a “cardioprotective state”. On the other hand, the clinical implication could be limited because many conditions, diseases and pharmaceuticals interfere with cardioprotection. In this thesis, we described that the protective potency of cardiprotective interventions is reduced by aging, diabetes, and hyperglycemia. It is known that many other conditions or pharmaceuticals can prevent preconditioning and postconditioning. A detailed understanding of these interactions is required to develop strategies to protect the myocardium of more patients.

Future Research

Not only the understanding of the exact mechanisms of preconditioning and postconditioning,, but also that of how pathological conditions, diseases and

pharmaceuticals abolish cardioprotection is incomplete. To us, the results described in this thesis demonstrate that mitochondria are critically involved in the signal transduction pathways of preconditioning and postconditioning. Furthermore, this thesis supports the hypothesis that mitochondrial mechanisms might be involved in the underlying mechanisms of the age and disease related reduction of the cardioprotective potency of preconditioning and postconditioning. Future studies should investigate whether mitochondria are possible targets for pharmacological therapy to restore cardioprotection.

REFERENCES

1. **Abete P, Ferrara N, Cioppa A, Ferrara P, Bianco S, Calabrese C, Cacciatore F, Longobardi G and Rengo F**. Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiol* 27: 1777-1786, 1996.
2. **del Valle HF, Lascano EC, Negroni JA and Crottogini AJ**. Absence of ischemic preconditioning protection in diabetic sheep hearts: role of sarcolemmal K_{ATP} channel dysfunction. *Mol Cell Biochem* 249: 21-30, 2003.
3. **Donnan PT, Boyle DI, Broomhall J, Hunter K, MacDonald TM, Newton RW and Morris AD**. Prognosis following first acute myocardial infarction in Type 2 diabetes: a comparative population study. *Diabet Med* 19: 448-455, 2002.
4. **Duan J and Karmazyn M**. Relationship between oxidative phosphorylation and adenine nucleotide translocase activity of two populations of cardiac mitochondria and mechanical recovery of ischemic hearts following reperfusion. *Can J Physiol Pharmacol* 67: 704-709, 1989.
5. **Ebel D, Mullenheim J, Frassdorf J, Heinen A, Huhn R, Bohlen T, Ferrari J, Sudkamp H, Preckel B, Schlack W and Thamer V**. Effect of acute hyperglycaemia and diabetes mellitus with and without short-term insulin treatment on myocardial ischaemic late preconditioning in the rabbit heart in vivo. *Pflugers Arch* 446: 175-182, 2003.
6. **Halestrap AP, Clarke SJ and Khaliulin I**. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 1767: 1007-1031, 2007.
7. **Hassouna A, Loubani M, Matata BM, Fowler A, Standen NB and Galinanes M**. Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium. *Cardiovasc Res* 69: 450-458, 2006.
8. **Hausenloy DJ and Yellon DM**. Preconditioning and postconditioning: united at reperfusion. *Pharmacol Ther* 116: 173-191, 2007.
9. **Hausenloy DJ, Yellon DM, Mani-Babu S and Duchen MR**. Preconditioning protects by inhibiting the mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol* 287: H841-H849, 2004.
10. **Juhaszova M, Rabuel C, Zorov DB, Lakatta EG and Sollott SJ**. Protection in the aged heart: preventing the heart-break of old age? *Cardiovasc Res* 66: 233-244, 2005.
11. **Katakam PV, Jordan JE, Snipes JA, Tulbert CD, Miller AW and Busija DW**. Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. *Am J Physiol Regul Integr Comp Physiol* 292: R920-R926, 2007.
12. **Kersten JR, Schmeling TJ, Orth KG, Pagel PS and Warltier DC**. Acute hyperglycemia abolishes ischemic preconditioning in vivo. *Am J Physiol* 275: H721-H725, 1998.

13. **Lee TM, Su SF, Chou TF, Lee YT and Tsai CH**. Loss of preconditioning by attenuated activation of myocardial ATP-sensitive potassium channels in elderly patients undergoing coronary angioplasty. *Circulation* 105: 334-340, 2002.
14. **Mullenheim J, Ebel D, Frassdorf J, Preckel B, Thamer V and Schlack W**. Isoflurane preconditions myocardium against infarction via release of free radicals. *Anesthesiology* 96: 934-940, 2002.
15. **Murphy E and Steenbergen C**. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* 88: 581-609, 2008.
16. **Murry CE, Jennings RB and Reimer KA**. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-1136, 1986.
17. **O'Rourke B**. Evidence for mitochondrial K^+ channels and their role in cardioprotection. *Circ Res* 94: 420-432, 2004.
18. **Pagel PS, Krolikowski JG, Shim YH, Venkatapuram S, Kersten JR, Weihrauch D, Warltier DC and Pratt PF, Jr.** Noble gases without anesthetic properties protect myocardium against infarction by activating prosurvival signaling kinases and inhibiting mitochondrial permeability transition in vivo. *Anesth Analg* 105: 562-569, 2007.
19. **Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV and Downey JM**. Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. *Circ Res* 87: 460-466, 2000.
20. **Palmer JW, Tandler B and Hoppel CL**. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem* 252: 8731-8739, 1977.
21. **Sato T, Saito T, Saegusa N and Nakaya H**. Mitochondrial Ca^{2+}-activated K^+ channels in cardiac myocytes: a mechanism of the cardioprotective effect and modulation by protein kinase A. *Circulation* 111: 198-203, 2005.
22. **Shanmuganathan S, Hausenloy DJ, Duchen MR and Yellon DM**. Mitochondrial permeability transition pore as a target for cardioprotection in the human heart. *Am J Physiol Heart Circ Physiol* 289: H237-H242, 2005.
23. **Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG and Jiang MT**. Cardiac mitochondrial preconditioning by Big Ca^{2+}-sensitive K^+ channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 290: H434-H440, 2006.
24. **Tani M, Suganuma Y, Hasegawa H, Shinmura K, Ebihara Y, Hayashi Y, Guo X and Takayama M**. Decrease in ischemic tolerance with aging in isolated perfused Fischer 344 rat hearts: relation to increases in intracellular Na^+ after ischemia. *J Mol Cell Cardiol* 29: 3081-3089, 1997.
25. **Tsang A, Hausenloy DJ, Mocanu MM, Carr RD and Yellon DM**. Preconditioning the diabetic heart: the importance of Akt phosphorylation. *Diabetes* 54: 2360-2364, 2005.

SUMMARY

Chapter 1 is the introduction of this thesis and presents a short overview over cardioprotective interventions. The phenomena of preconditioning and postconditioning, and main components of their respective underlying mechanisms with a focus on the role of mitochondria in cardioprotection are described.

In **Chapter 2,** we investigate the bioenergetic consequences of mK_{Ca} channel activation in isolated heart mitochondria. Our results demonstrate that matrix K^+ influx by mK_{Ca} channel activation increased the rate of mitochondrial respiration in a dose-dependent manner. Mitochondrial reactive oxygen species production was markedly increased due to maintenance of a high resting membrane potential in the face of markedly enhanced respiration. From our data we suggest a mechanism by which reactive oxygen species can be generated by K^+ influx into the mitochondrial matrix due to mK_{Ca} channel activation.

The impact of mK_{Ca} channel activation on reversed electron flow-induced reactive oxygen species is investigated in **chapter 3**. We demonstrate that mK_{Ca} channel activator NS1619 reduces mitochondrial reactive oxygen species production that are generated by reversed electron flow into complex I of the electron transport chain. The relative decrease in reverse electron flow, and thereby free radical generation, is likely due to increased forward electron flow due to stimulated mitochondrial respiration.

The protective potency of ischemic preconditioning has been shown to be reduced in the aged myocardium. In **chapter 4**, we demonstrate that the effect of mK_{Ca} channel activation, which is critically involved in ischemic preconditioning, is abolished in mitochondria that were isolated from aged rat hearts. We concluded that this might be one underlying reason of the decreased protective potency of ischemic preconditioning in the aged myocardium.

Pharmacological preconditioning can be initiated by the noble gas helium. In **chapter 5**, we investigated whether mK_{Ca} channel activation and regulation in mitochondrial respiration is involved in helium-induced preconditioning. We demonstrate that helium confers preconditioning via mK_{Ca} channels and regulates mitochondrial respiration. Furthermore, both the cardioprotective properties and the effect on mitochondrial respiration are age-dependent.

In **chapter 6**, we investigate whether helium induces preconditioning in the pre-diabetic myocardium. We demonstrate that helium-induced preconditioning is abolished in obese Zucker rats *in vivo* probably. This reduction in protective potency is associated with by a diminished effect of Helium on mitochondrial respiration.

As described in chapter 5 and 6 of this thesis, the noble gas helium induced early myocardial preconditioning in the rat *in vivo*. We investigated in **chapter 7** whether helium induces also late preconditioning, and if so, whether the cardioprotective effect is concentration-dependent. Furthermore, we studied whether repeated administration of helium on subsequent days will result in an increased cardioprotection. We could show that helium induces late preconditioning and that cardioprotection is already maximal with administration of one cycle of 30% helium.

Chapter 8 focuses on the interaction of hyperglycemia and sevoflurane-induced postconditioning. We demonstrate that hyperglycemia abolishes the protective effect of sevoflurane-induced postconditioning, but pharmacological inhibition of the mPTP by cyclosporine A could restore the cardioprotective effect.

The initial aim of the study described in **chapter 9** was to investigate whether inhibition of the mPTP is involved in morphine-induced preconditioning in isolated rat hearts, but the expected cardioprotection was not detectable. Thus, we investigated the influence of different preconditioning protocols and substrate conditions on cardioprotection in this experimental model. We demonstrated that physiological levels of glutamine prevented morphine-induced preconditioning.

In **chapter 10**, the main conclusions of this thesis are summarized. Furthermore, we included a general discussion about clinical implications and limitations of experimental models in this chapter.

NEDERLANDSE SAMENVATTING

Hoofdstuk 1 vormt de inleiding van dit proefschrift en geeft een kort overzicht van cardioprotectieve interventies. De fenomenen preconditioning en postconditioning worden beschreven, alsmede de belangrijkste elementen van hun onderliggende mechanismen met een nadruk op de rol van het mitochondrion.

In **hoofdstuk 2** onderzoeken we het gevolg van mK_{Ca} kanaalactivatie op het mitochondriële energiemetabolisme in het geïsoleerde hart. Onze resultaten tonen aan dat matrix K^+ influx door mK_{Ca} kanaalactivatie de snelheid van mitochondriële zuurstofconsumptie doet toenemen op een dosis afhankelijke wijze. De mitochondriële productie van reactieve zuurstofsoorten werd duidelijk verhoogd indien een hoog transmembraanpotentiaal werd gehandhaafd in combinatie met een verhoogde zuurstofconsumptie. Onze data doet vermoeden dat de productie van reactieve zuurstofsoorten wordt veroorzaakt door K^+ influx de mitochondriële matrix in door mK_{Ca} kanaalactivatie.

De impact van mK_{Ca} kanaalactivatie op de productie van reactieve zuurstofsoorten die door omgekeerde flux van elektronen ontstaan, wordt onderzocht in **hoofdstuk 3**. We laten zien dat de mK_{Ca} kanaal activator NS1619 de mitochondriële productie van reactieve zuurstofsoorten vermindert, die ontstaan door omgekeerde flux van elektronen naar comlex I van de elektronentransportketen. De relatieve afname van de omgekeerde elektronenflux en daarbij de productie van vrije radicalen, is waarschijnlijk het gevolg van een toename in de voorwaartse elektronenflux door verhoogde mitochondriële zuurstofconsumptie.

Het is aangetoond dat het beschermend vermogen van ischemische preconditioning verminderd is in het oude myocardium. Activatie van mK_{Ca} kanaal is een kritieke stap in ischemische preconditioning. In **hoofdstuk 4** laten we zien dat het effect van mK_{Ca} kanaalactivatie te niet gedaan is in mitochondria die geïsoleerd waren uit de harten van oude ratten. Wij concluderen dat dit mogelijk een van de onderliggende mechanismen is van het verminderd beschermend vermogen van ischemische preconditioning in het oude myocardium.

Het edelgas helium kan farmacologische preconditionering in werking stellen. In **hoofdstuk 5** onderzoeken wij of mK_{Ca} kanaalactivatie en regulatie in mitochondriële zuurstofconsumptie betrokken is bij helium-geïnduceerde preconditioning. Wij laten zien dat helium preconditionering via mK_{Ca} kanaalactivatie geeft, en mitochondriële zuurstofconsumptie reguleert. Zowel de cardioprotectieve eigenschappen als het effect op de mitochondriële zuurstofconsumptie zijn leeftijdsafhankelijk.

In **hoofdstuk 6** onderzoeken wij of helium preconditioning induceert in het pre-diabetisch myocardium. Wij tonen aan dat helium-geïnduceerde preconditioning te niet gedaan wordt in obese Zucker ratten *in vivo*.
Deze reductie in beschermend vermogen is geassocieerd met een verminderd effect van helium op mitochondriële zuurstofconsumptie.
Zoals beschreven in hoofdstuk 5 en 6 van dit proefschrift, induceert helium vroege myocardiale preconditioning in de rat *in vivo*. In **hoofdstuk 7** onderzoeken wij of helium ook late preconditioning induceert, en indien dit het geval is, of het cardioprotectieve effect concentratie afhankelijk is. Bovendien hebben we gekeken of herhaaldelijke toediening van helium op opeenvolgende dagen, een verhoogde cardioprotectie geeft. We hebben aangetoond dat helium late preconditioning induceert en dat maximale cardioprotectie reeds bereikt wordt bij toediening van een cyclus van 30% helium.
Hoofdstuk 8 richt zich op de interactie van hyperglycemie en sevofluraan-geïnduceerde postconditioning. Wij laten zien dat hyperglycemie de beschermende werking van sevofluraan-geïnduceerde postconditioning te niet doet. Farmacologische inhibitie van mPTP door cyclosporine A kan de cardioprotectieve werking van sevolfluraan echter herstellen.
Het oorspronkelijk doel van de studie die beschreven staat in **hoofdstuk 9**, was te onderzoeken of de inhibitie van de mPTP betrokken is bij morfine-geïnduceerde preconditioning in geïsoleerde rattenharten. De verwachte cardioprotectie was echter niet aantoonbaar. Daarom hebben wij onderzocht wat de invloed van verschillende preconditioning protocollen en substraat condities is op cardioprotectie in dit experimentele model. Wij tonen aan dat fysiologische waarden van glutamine morfine-geïnduceerde preconditioning voorkomt.
In **hoofdstuk 10** worden de voornaamste conclusies van dit proefschrift samengevat. Een algemene discussie over de klinische implicaties en de beperkingen van de experimentele modellen zijn ook opgenomen in dit hoofdstuk.

ACKNOWLEDGMENT

Ich bedanke mich bei meinen Promotoren Herrn Prof. Dr. Dr. Markus W. Hollmann und Herrn Prof. Dr. Wolfgang Schlack für das von ihnen in meine Person gesetzte Vertrauen und für die Möglichkeit, bei ihnen promovieren zu können.

Es liegt mir am Herzen, mich sowohl bei Herrn Prof. Dr. Volker Thämer als auch bei Herrn Dr. Jost Müllenheim dafür zu bedanken, dass sie während meines Studiums meine Begeisterung für physiologische Grundlagenforschung geweckt haben.

Dr. Stowe, Amadou, Mohammed, Matthias, Anita, Samhita, James and Gopu, it is extremely difficult to find words that roughly depict my year in Milwaukee. I feel deeply grateful for everything that I was allowed to experience: meeting great people, bright researchers, spending hours of discussions about a broad range of topics (including mitochondrial bioenergetics, George W. Bush, and the Gambian River Journal). If you want to know whether I miss this time, please feel free to ask Antje or my friends what happens to me when I am talking about this year.

Mein Dank gilt meinen Co-Promotoren Frau Dr. Nina C. Hauck und Herrn Dr. Benedikt Preckel für die Betreunung meines PhD -Projektes während meiner Zeit in Düsseldorf und Amsterdam.

Ragnar and Gitara, thanks for everything. Without M0-126, this book would not exist. I hope that both of you know how deeply grateful I am.

Coert, het is heel jammer dat jij niet voor mij hebt gekozen als student voor jouw hexokinase project. Ik ben absoluut zeker dat ik dat geweldig zou hebben gevonden. Maar ook zo, hartelijk dank voor alle wetenschappelijke discussies en alle steun die ik van jou heb gekregen.

I would like to thank the L.E.I.C.A. laboratory team for supporting me, in particular Jan, Kirsten, Raphaela, Anneke, Jorrit and Alexander.

Von Herzen danke ich meiner Familie und meinen Freunden für ihre nicht abreißende Unterstützung.

ABOUT THE AUTHOR

André Heinen studied medicine at the University of Düsseldorf, Germany (1997-2003). After his graduation, he started his residency at the Department of Clinical Anaesthesiology (Prof. Dr. J. Tarnow), University Hospital Düsseldorf, Germany. Subsequently, he worked as research fellow at the Department of Anesthesiology at the Medical College of Wisconsin, Milwaukee, USA, and completed his PhD-thesis at the Laboratory of Experimental Intensive Care and Anesthesiology (L.E.I.C.A.), Department of Anesthesiology at the University of Amsterdam, the Netherlands. He published widely in the field of mitochondrial bioenergetics and cardioprotection against ischemia and reperfusion injury.