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Nitric oxide in focal cerebral ischemia, an experimental study

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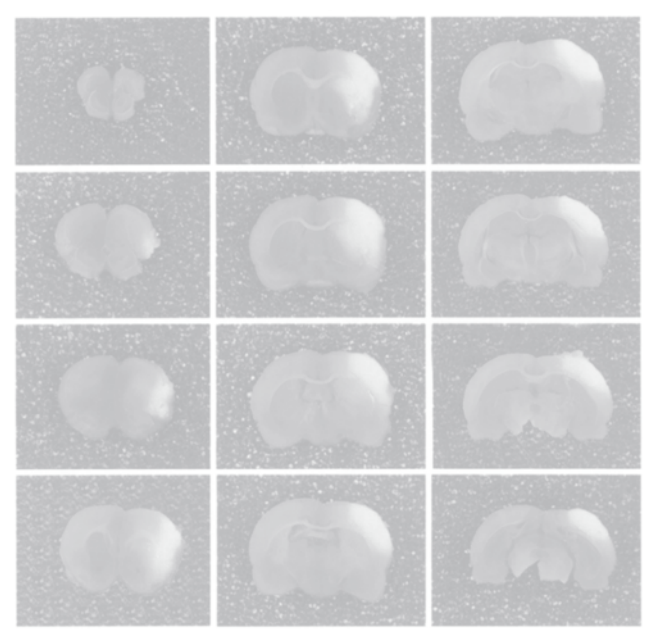
Chapter 8

Summary
Samenvatting



SUMMARY

In vivo models have been an important tool to study the pathophysiology of focal cerebral ischemia and test possible protective strategies. The rat MCA occlusion model is a frequently used model to study focal cerebral ischemia; it simulates ischemia during temporary arterial occlusions in neurovascular procedures. In rat MCA occlusion models volume and reproducibility of infarction were found to depend on duration of occlusion[9], anesthetic technique[39], temperature[15], bloodpressure[46], serum glucose levels[38], strain[19,29], and histologic technique. The interactions between these factors can be illustrated by the effects of the anesthetics halothane and isoflurane on cerebral ischemia[39]. The protective effects of these anesthetics are attributed to effects on metabolism. In higher doses anesthetics can increase infarct volume by reducing blood pressure and consequently collateral flow. Detailed appraisal of physiologic parameters like brain temperature and blood pressure combined with information on anesthetic technique can help interpret results in experimental stroke. Overall the severity of ischemia can be increased by extending the duration of occlusion and by reducing collateral flow. Using the Tamura model, a focal cerebral ischemia model in the rat, after 1 hour MCA occlusion 60% of animals did not develop any cortical infarction (chapter 2)[9]. The addition of bilateral common carotid occlusion reduced this to 25 %. Extending the occlusion time to three hours resulted in an improvement (chapter 2)[9]. Cortical infarct volumes with 3 hours of MCA and bilateral CCA occlusion equaled values found in permanent MCA occlusion (chapter 2)[9]. The MCA stroke did not cause severe neurologic deficits[41], but this MCAo technique caused temporo-mandibular dysfunction interfering with the ability of the animal to eat. Reperfusion was limited to three days to limit weight loss, which averaged 24% after 7 days. Reproducibility is a major concern in models of cerebral ischemia. Efforts to improve reproducibility will reduce the number of animals required. Increasing the severity of cerebral ischemia increased average infarct volume and reduced variability of results. In our experiments 3 hours of MCA occlusion with bilateral CCA occlusion produced cortical infarcts of 83 mm^3 (average) in the male Wistar rat under (1%) Halothane anesthesia and normocapnic, normotensive, normothermic and normoxic conditions.

Studies on the cascade of events during cerebral ischemia have resulted in the identification of the glutamate activated NMDA receptor mediated transmembrane calcium influx[8]. The activation of calcium dependent enzymes mediates the deleterious effects down stream. The Nitric Oxide Synthase (NOS) iso-enzymes were identified as important calcium dependent enzymes[34] located in neurons (nNOS), endothelium (eNOS) and glial cells and macrophages (iNOS). The importance of the discovery of nitric oxide as a signaling molecule in the cardiovascular system was confirmed in 1998 when the Nobel Price was awarded. Under pathological circumstances, like ischemia, the NOS system is

overly activated and the excessive concentrations of NO are an important mediator of NMDA toxicity[34]. Because the half-life of NO is short, direct measurements are difficult. Direct and indirect measurements of NO using different methods have identified peak levels of NO early after the onset of ischemia[23,26]. Indirect indications on the role of NO in cerebral ischemia were derived from studies with NOS inhibitors and NO donors. The effects of non iso-enzyme specific NOS inhibitors on ischemic cerebral injury have been contradictory and a dose-dependent dual role was proposed[5]. More consistent results were found using iso-enzyme selective NOS inhibitors. Nonspecific side effects of these drugs were excluded using knock out animals for NOS iso-enzymes. The nNOS knock-out animals, developed smaller infarcts which confirms the deleterious effects of NO found in in vitro studies[10]. On the other hand NO donors were found to reduce ischemic damage in vivo if hypotension was avoided[44] . Direct comparison of effects of NOS inhibition and NO donors however was complicated by important differences in methodology between investigators. In a model that simulates ischemia during temporary arterial occlusions in neurovascular procedures two NOS inhibitors and two NO donors were tested for dose-dependent neuroprotective effects. Non-selective NOS inhibitor L-NAME reduced the cortical infarct volume significantly in the medium (1.0 mg/kg) dose group while the selective nNOS inhibitor 7-NI was significantly protective in the higher (10 and 100 mg/kg) dose groups. Detrimental effects of higher dose L-NAME treatment were attributed to the effect on eNOS causing a reduction in collateral flow. When compared to the appropriate control group 7-NI reduced infarct volume up to 92% (chapter 3, fig 1) to which the anesthetic effect of 7-NI may have contributed.

The anti-ischemic effects of NO donors were recognized for coronary artery disease for more than a century[7] and are used to date in acute coronary syndromes. In our model of focal cerebral ischemia with 3 hours of MCA and bilateral CCA occlusion treatment with both NO donors reduced the average cortical infarct volume but this reduction did not reach significance (chapter 3, fig 1).

Polyamines with NO donor capacity were previously reported to reduce ischemic injury in experimental focal cerebral ischemia[33]. Under physiologic conditions Polyamines like spermine, putrescine and spermidine were found to regulate important cellular functions like Ca^{2+} transport[21,25] and nitric oxide synthase[20] and free-radical scavenging[21]. The exact role of polyamines in the pathophysiology of stroke is unclear. Reductions of spermine levels were reported in experimental focal cerebral ischemia[31,35]. Treatment with spermine resulted in reduction of ischemic damage in experimental forebrain ischemia[13]. To evaluate protective efficacy of the spermine part and to enable comparison to previous experiments using NOS inhibitors and NO donors, the exact same 3 hours tandem MCA and CCA occlusion model was used. In the 10 mg/kg i.v. spermine treatment group infarct volume was significantly reduced when compared to controls. In the

lower dose treatment groups the reduction in infarct volume was not significant. This effect was stronger than the effect of the NO donors used in previous studies (chapter 3). Treatment with spermine resulted in a significant decrease in serum glucose levels of about 30 % and a dose-dependent decrease in serum lactate. Mechanisms proposed to explain the protective effects of spermine include hypoglycemia and direct effects on NMDA receptor[28] and nNOS inhibition[20]. The protective effects of spermine (10 mg/kg) approximate the effects of selective nNOS inhibition (7-NI, 10 mg/kg). Studies with combinations of spermine and NOS inhibitors and/or NO donors may help to determine the exact mechanisms.

Selective deficiency or inhibition of the neuronal NOS enzyme was found to reduce cerebral infarct volume while for non iso-enzyme specific NOS inhibitors more complex dosage and time dependent effects have been described[27,45]. The finding that the severity of the ischemic insult determined the neuroprotective efficacy of NOS inhibitors[2,16] and reports of biphasic pH sensitivity of the NOS enzyme[14,24,32] led to the hypothesis that the neuroprotective effects of (n)NOS inhibition are dependent on ischemic intracellular pH. Manipulation of serum glucose is a well-documented method to alter brain pHi during cerebral ischemia[4]. Hyperglycemia may have additional effects on the extent of ischemic damage not mediated through the intracellular pHi however. Hyperglycemia (serum glucose levels of 20 mmol/l) was used during experimental focal cerebral ischemia to exacerbate and moderate hypoglycemia (serum glucose level 3 - 5 mmol/l) to attenuate intracellular acidosis. This resulted in an average infarct volume of 170 mm^3 for hyperglycemia (77% increase) and 19 mm^3 for moderately hypoglycemia (80% decrease). In vivo fluorescence imaging techniques were used to study pHi with rCBF (regional cerebral blood flow) and the NAD+/NADH ratios[3]. For the in vivo fluorescent imaging the occlusion time was limited to two hours, which resulted in an adjustment of the experimental protocol. This resulted in an average cortical infarct volume that was not significantly different from the 3 hour MCA and CCA occlusion protocol. During normoglycemic (average serum glucose 9 mmol/l) ischemia pHi declined from 7.0 to 6.6. After release of the MCA and CCA occlusions, rCBF and pHi recovered. Hyperglycemia (serum glucose of 20 mmol/l resulted in a decline of pHi from 7.0 to 6.1. Under moderate hypoglycemic (serum glucose 5 mmol/l) conditions pHi reached 6.8. Under hyperglycemic conditions selective nNOS inhibition with 7-NI 100mg/kg resulted in a 28% reduction of the infarct volume, while under normoglycemic conditions this reduction was 93%. Moderate hypoglycemia resulted in a 73% reduction. Based on in vitro studies of pH sensitivity of the NOS enzyme[14,17,32] within the observed pHi range (6.1 to 6.8) enzyme activity would vary considerably. With Citruline measurements the expected subsequent increase in NOS activity on restoration of pHi was observed by Wei and Quast [40]. The results of our study

support the hypothesis that the neuroprotective efficacy of selective nNOS inhibition is dependent of the intracellular pHi.

The beneficial effects of acute treatment with NO donors has previously been reported[42-44]. NO donors like SIN-1 are used in interventional cardiology for their antispastic and vasodilatory effects[36]. The NO release capacity is affected by pO_2 and pH but adequate under ischemic conditions[6]. The use of the single shot NO donor resulted in a temporary blood pressure reduction with spontaneous recovery within 30 min (which is before occlusion). To investigate the relationship between intracellular pH and the role of NO in focal cerebral ischemia, moderate hypoglycemia, normoglycemia or hyperglycemia was created. The effect of treatment with an NO donor was evaluated under these conditions. As in the previous chapter in vivo fluorescence imaging data was used for pHi, rCBF and the NAD+/NADH ratios[3]. For the in vivo fluorescent imaging the occlusion time was limited to two hours (as explained in chapter 6). As presented in chapter 6 during normoglycemic ischemia pHi declined from 7.0 to 6.6. After release of the MCA and CCA occlusions the pHi recovered to 6.7. Hyperglycemia (serum glucose levels 20 mmol/l resulted in a decline of pHi from 7.0 to 6.1. Under moderate hypoglycemic (serum glucose 5 mmol/l) conditions pHi reached 6.8. In our study SIN-1 did not affect serum glucose levels (chapter 7, table 1). Under normoglycemic and hyperglycemic conditions the NO donor SIN-1 significantly reduced cortical infarct volume, this in contrast with previous results with the 3 hour schedule when the reduction did not reach significance (chapter 3, fig 1D). The reduction of infarct volume for the SIN-1 treated animals under moderately hypoglycemic condition was not significant (chapter 6, fig 2). When these results are combined with data on pH sensitivity of the NOS enzyme our results indicate that under acidotic circumstances in which the NOS enzyme is less active the protective effects of exogenous NO is retained.

Detailed appraisal of experimental settings and their effects on outcome can increase the reproducibility of experimental models. Changes in variables like occlusion time, collateral flow and serum glucose levels lead to important differences in infarct size. The translation of evidence from the basic science lab into the clinic has proven difficult[37]. Attention was demanded for pharmacokinetics, dosing and choices of experimental models and outcome parameters[1]. This will hopefully lead to more success in the future. The complex role of NOS under physiologic circumstances and in the development of stroke and its limited therapeutic time window make it less suitable as a direct target for therapy. The evidence for an important contribution of NOS activation in the pathophysiology of stroke is convincing. To date the direct quantification of NO is difficult. NO donors and NOS inhibitors have made it possible to study the role of NO indirectly; bypassing direct measurements. The results of these indirect studies should be carefully interpreted

because effects of the used compounds may not only be NO related; as was illustrated by our spermine experiments. The reduced effectivity of nNOS inhibition in more severe ischemia was explained by the pH sensitivity of the NOS enzyme. More severe ischemia will result in more pronounced acidosis. Under these conditions the NOS enzyme is inhibited, explaining the limited effects of further inhibition. Neuronal NOS inhibition did not affect the marked increases in lactate in the post-ischemic brain or the recovery of other energy-related metabolites[18]. As expected, the protective effects of exogenous NO was preserved under acidotic conditions. This effect was recently confirmed for the NO donor sodium nitrite[22]. Focus has shifted from nNOS to the other iso-enzymes. In a study on gender differences in ischemic brain injury, estrogens' neuroprotective effects were related to the attenuation of iNOS expression[30]. Stroke and cardiovascular event protection associated with regular physical activity was found to be mediated by endothelial NOS upregulation[12]. The protective effects for Angiotensine II type 1 (AT-1) receptor inhibitor candesartan and HMG coA (3-hydroxy-3-methyl-glutaryl-coA) reductase inhibitor rosuvastatin were also found to be at least partly mediated by eNOS upregulation[11]. Increasing knowledge on this mechanism and the role NO in physiology and pathology will be a strong basis for the development of therapies in the future.

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