Mitochondrial mechanisms of perinatal hypoxic brain injury

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Summary

Cerebral hypoxic/ischemic injury is a relatively common occurrence in the perinatal period. Using animal models, we have been working on the pathomechanism of the disturbed cerebral blood flow and metabolism. During my 5 month fellowship we studied the role of the adenosine triphosphate (ATP) –sensitive K^+ channels (mitoK_{ATP}) found on the mitochondrial membrane in the cellular disturbances caused by hypoxia. We used cultured cell-lines, freshly isolated mitochondria from the brain and brains samples for the ex-vivo studies. The subjects of our in vivo studies were newborn piglets and adult rats. We found that diazoxide, a selective mitoK_{ATP} agonist depolarizes mitochondria and this effect is accompanied by production of reactive oxygen species. We also found that pretreatment with diazoxide inhibits the damage in blood brain barrier caused by hypoxic stress. In addition, we provided evidence that there are no functional N-methyl-D-aspartate receptors on the cerebral blood vessels derived from newborn pigs. We started new projects and generated several new ideas. I regard my fellowship very successful both on professional and personal level.

1. Background

Perinatal asphyxia - when a baby does not receive enough oxygen before, during or just after birth- occurs in approximately 6 per 1000 term live births and remains the most important cause of neurological injury in the newborn. During asphyxia, the neuronal homeostasis is severely disrupted by the rapidly developing hypoxia, hypercapnia and decreased cerebral blood flow (~global ischemia). These changes initiate a complex sequence of pathophysiological events, culminating in the malfunction of neurons, e.g. seizure activity or neuronal cell death. The mechanisms involved in mediating neuronal impairment in the neonate are complex and poorly understood.

Using severe, but clinically relevant periods of hypoxia, global cerebral ischemia or asphyxia in piglets – a widely used and accepted model for human babies- , we found that cortical vascular reactivity – the ability of the brain's vessels to dilate or constrict- is impaired selectively to various stimuli. A major part of the dilator responses of the cerebral blood vessels are suppressed. In addition, the neuronal-vascular coupling which is essential for the appropriate blood supply in the central nervous system is also damaged. Within hours after hypoxic-ischemic stress, characteristic changes in cerebral protein synthesis can be observed. Proteins promoting cell survival are induced as are factors delaying neuronal death. Because of its potential role in neuroprotection, the hypoxia/ ischemia-induced gene expression has been extensively studied. We have long been interested in detecting the early changes in expression of a variety of potentially beneficial or harmful proteins. We earlier demonstrated that global cerebral ischemia/reperfusion alters the levels of synthesis and expression of heat shock protein 72. - (Heat shock proteins (HSPs), also called stress proteins, are a group of proteins that are present in all cells in all life forms. They are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation. Heat shock proteins are also present in cells under perfectly normal conditions. They act like 'chaperones,' making sure that the cell's proteins are in the right shape and in the right place at the right time)-., endothelial nitric oxide synthase – (endothelial NOS is an enzyme which generates nitric oxide (NO) in blood vessels and is involved with regulating vascular function. A constitutive Ca2+ dependent NOS provides a basal release of NO) - and cyclooxygenase-2 – (cyclooxygenase (COX) is an enzyme that is responsible for formation of important biological mediators called prostanoids (including prostaglandins and thromboxane). Pharmacological inhibition of COX can provide relief from the symptoms of and pain; this is the method of action of well-known drugs such as aspirin and ibuprofen. Currently three isoenzymes of COX (-1, -2, -3) are known. Different tissues express varying levels of COX-1 and COX-2. Although both enzymes act basically in the same fashion, selective inhibition can make a difference in terms of side-effects. COX-1 is considered a constitutive enzyme, being found in most mammalian cells. COX-2,

on the other hand, is undetectable in most normal tissues. It is an inducible enzyme, becoming abundant in activated macrophages) as early as 2-6 hrs after hypoxic stress.

One component of the original insult constituting anoxia/ reoxygenation may be the damage caused by oxygen radicals. - A prominent feature of radicals is that they have extremely high chemical reactivity, which explains not only their normal biological activities, but how they inflict damage on cells. We and others have demonstrated that a significant source of superoxide anion is the impaired respiratory chain in the mitochondria. We have shown that cerebrovascular dysfunction caused by transient anoxic stress can be prevented by administration of oxygen radical scavengers such as superoxide dismutase.

The purpose of my Fulbright Award application was to make it possible for me to work in Dr. David W. Busija's laboratory at the Wake Forest University School of Medicine, in Winston-Salem, North Carolina and to continue and extend our productive, collaborative relationship. While Dr. Busija had NIH and AHA funds to support the major parts of my research plan, the fund was necessary to pay for my salary and partially covered my travel costs.

The purpose of the research effort was to extend our previous, original observations on mechanisms of neuroprotection against anoxic injury in the neonatal pig. This is an important area of research because of the relatively high frequency of hypoxia-ischemic stress during the perinatal period, which leads to death of babies or to pathologies such as cerebral palsy. The piglet model for studying perinatal hypoxic brain injuries is widely used.

In 1999, Dr. Busija and myself made the first observation in the brain showing that potassium channels in mitochondria were a potential therapeutic target for protection of neurons against ischemic stress. Thus, using diazoxide, a drug that specifically targets the ATP-sensitive channels in mitochondria (mitoK_{ATP} channels), we were able to completely protect neuronal function against 10 minutes of complete brain ischemia. Ischemia was induced by stopping blood flow to the brain by increasing the intracranial pressure. Intactness of neuronal function was tested using an analogue of glutamate, namely N-methyl-D-aspartate (NMDA), which targets specifically one of the glutamate receptor subtypes. This in vivo assay is extremely sensitive and detects even minor disturbances in the neuronal-vascular coupling.

While this was an important first step, there was a need to develop new techniques to study this phenomenon more extensively in the newborn pig. We have focused on this model rather than rodent species because of the similarity of developmental status of the piglet and human baby brains. Unfortunately, study of the responses of the piglet brain to ischemia is difficult because of previously existing technical problems. Dr. Busija and myself have spent the last several years independently developing new approaches. At the time of the Fulbright grant application, in 2002, we believed that we were in a

position to combine these approaches in new, original experiments designed to determine the mechanisms involved in neuroprotection induced by activation of mitoK$_{ATP}$ channels. However, since these approaches were complex, it was necessary for us to collaborate, at least initially, via direct involvement in the same laboratory.

While it is well recognized in the heart that mitochondria are a potential target for pharmacological agents in the prevention of cellular damage following ischemic stress, little work has been done in the brain because of technical limitations. Following our initial observation, we have focused on overcoming these technical limitations and at that time we were in a position to continue our studies. The most important advances made by us are as follows: together with my Hungarian coworkers (Drs. Siklos and Domoki, Szeged) I have developed a method for assessing changes in calcium levels in piglet mitochondria following ischemic stress in piglets. This method involves the use of electron microscopy and a reagent that binds to calcium. In this provocative study, we showed that neuroprotection in piglets is associated with a limitation of calcium influx into mitochondria. An influx of free calcium to mitochondria is thought to be a crucial first step in causing immediate damage to mitochondria with subsequent (necrotic or apoptotic) death of neurons. However, until this recent study there was no direct evidence that activation of mitoK$_{ATP}$ channels could limit calcium influx following ischemia. In complementary experiments, Dr. Busija has showed that activation of mitoK$_{ATP}$ channels is able to limit infarct volume in adult rats and rat pups in different models of ischemia. Further, Dr. Busija has developed cell culture methods for neurons and astroglia to address the mechanisms of protection via activation of mitoK$_{ATP}$ channels. Additionally, Dr. Busija has developed methods to characterize effects of activation of mitoK$_{ATP}$ channels on mitochondria membrane potential and oxygen radical production using confocal microscopy.

Based upon our observations and information from the literature, we developed the overall hypothesis and specific aims.

2. Realization of our plans

Of course there was a considerable time-gap between the grant application and the realization of my trip to North Carolina. After the positive decision of the Fulbright Board we made more specific plans. I started my work on the 15th of July in 2003 and finished exactly 5 months later.

With the help of Dr. Busija and his coworkers we conducted several experiments and started new ones. I spent lot of time in the lab. I started with work at 8:00 am and left around 6-7 pm. During the 5 months period I wanted to explore as many problems as possible. After awhile I had to realize that I was unable to complete the studies I started with. I knew that the goals were appropriate and the approaches also reasonable. Since I left the lab we were able to finish almost all the studies. Furthermore, we were able to publish our results in well respected journal. Now I would like to summarize the most results of the most important studies.

2.1. Study #1:

The purpose of this study was to examine whether the effects of diazoxide on mitochondrial membrane potential ($\Delta\Psi_m$) and ROS – reactive oxygen species-production were due to the activation of mitoK$_{ATP}$ channels or other effects, such as on succinate dehydrogenase (SDH) inhibition. In isolated piglet mitochondria, we compared effects of diazoxide with a newly developed mitoK$_{ATP}$ channel opener, BMS-191095, which does not increase ROS production in cultured neurons and apparently does not inhibit SDH. Additionally, we administered 3-nitropropionic acid (3-NPA), a specific inhibitor of SDH, which appears to have no direct effect on mitoK$_{ATP}$ channels.

We used brains from neonatal pigs (1–7 days of age) to isolate mitochondria. Using confocal laser microscopy we investigated the mitochondria in various conditions. Reactive oxygen species generation was assessed using the oxidation-sensitive dye dyhydroethidum (HEt, Molecular Probes, Eugene, OR, USA), which is oxidized to the fluorescent ethidium by free radicals. The major finding of the study was that while both diazoxide and BMS-191095 depolarize mitochondria, diazoxide but not BMS-191095 promotes ROS production. It seems likely that enhanced ROS production is due to SDH inhibition, a well known characteristic of diazoxide, since 3-NPA leads to ROS production independent of changes in $\Delta\Psi_m$. Thus, ROS production by mitochondria is not necessarily linked to mitoK$_{ATP}$ channel opening and neuroprotection.

2.2. Study #2:

The blood brain barrier (BBB) is responsible for the limited and regulated movement of plasma constituents into the brain parenchyma. During pathophysiological conditions, damage to endothelial cells and alterations in BBB function can have adverse effects on the brain. BBB disruption was found to precede and may be the initiating event of focal ischemic lesions in hypertensive encephalopathy [38]. BBB injury can worsen the outcome after cerebral ischemia as well [19]. Therefore, it is important to develop approaches to limit BBB dysfunction after cerebral ischemia. One possibility is to use specific drugs which induce protection against the expected hypoxia/ischemia. When a drug is used in a very low concentration and the beneficial action is still present after the complete clearance from the drug the effect is regarded as preconditioning. The exact underlying mechanisms of the preconditioning are unknown; however, it can be triggered by a variety of other stimuli, including heat shock, exercise, opioids and cytokines. In addition, activation of mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels has been proposed to play a pivotal role in preconditioning. Pharmacological agents that open mitoK$_{ATP}$ channels reproduce preconditioning without any other intervention. Moreover, physiological or chemical preconditioning is prevented by blockers of the mitoK$_{ATP}$ channels. The beneficial effects of the prototype mitoK$_{ATP}$ channel opener diazoxide have been well demonstrated in the heart and other organs and

have been shown in experimental neurological preparations by our laboratory and by others. The purpose of our study was to determine whether diazoxide pretreatment affects BBB function following ischemic stress in rats. We tested the hypothesis that diazoxide would reduce BBB permeability and decrease brain water content following I/R, and that mitoK_{ATP} channel opening would be involved in its action.

Experiments were carried out on male rats. The animals were exposed to global cerebral ischemia using combined bilateral common carotid artery occlusion and arterial hypotension. Blood-brain barrier permeability was assessed by measurement of Evans blue and sodium fluorescein content in brain.

The main findings of our experiments are that diazoxide treatment applied in a preconditioning protocol (3 consecutive days before ischemia) gave protection against BBB opening and brain oedema in a severe global ischemic model in rat. Diazoxide can depolarize the mitochondria of brain endothelial cells, raising the possibility of direct preconditioning of these cells.

2.3. Study #3:

Dr. Busija and his coworkers were the first to report that glutamate and its synthetic analogue N-methyl-D-aspartate (NMDA) are dilator agents in the *in vivo* piglet cerebral circulation. These original studies in piglets have been replicated in other species such as rat and rabbit. The mechanism of dilation is not completely understood, but probably involves activation of neuronal NMDA receptors, generation of nitric oxide and/or other substances, and subsequent actions on arterioles of these vasoactive factors. Recently, the view that arteriolar dilation in the cerebral circulation to NMDA is secondary to neuronal release and vascular actions of NO or other brain-derived substances has been challenged.

The purpose of this study was to compare vascular responses *in vivo* and *in vitro* to NMDA in the piglet cerebral circulation. The *in vitro* experiments were done by two independent laboratories at four different intravascular pressures, and intactness of vascular function was assessed with bradykinin. Bradykinin is an endothelium-dependent dilator agent in piglets. Additionally, we examined whether glutamate dilated isolated piglet cerebral arteries.

We worked on isolated arteriolar branches of piglet middle cerebral artery. Branches from both middle cerebral arteries (MCAs) were carefully harvested using a dissecting microscope. These arteriolar branches of the MCA (about 2 mm in length) were transferred to a vessel chamber, mounted, and secured between two glass micropipettes with 10-0 ophthalmic suture. Vascular reactivity was then determined in one of two protocols done by each laboratory: Protocol 1 at 30 and 80 mmHg intraluminal pressures in Dr. Busija's laboratory and Protocol 2 at 60 and 100 mmHg intraluminal pressures in Dr. Eckman's laboratory (Wake Forest School of Medicine, Winston-Salem, NC).

The major finding of the study is that isolated piglet cerebral arteries fail to

dilate to NMDA while NMDA dilates arteries markedly *in vivo*. Isolated arteries were pressurized to four different levels, and experiments on isolated arteries were done in two independent laboratories. Similarly, we were unable to elicit significant dilator responses to glutamate in isolated arteries at doses up to 1mM. In contrast, isolated arteries dilated substantially to bradykinin, thereby establishing intactness of the preparation and viability of endothelial function. Thus, we cannot provide support for the concept that dilation occurs via direct actions of NMDA or glutamate on the vascular wall.

3. Conclusion

I initially expected to return to North Carolina simply to conduct research, but I left with so much more. I have gained a rare inside view into the complexities of scientific research and an international perspective on evaluating problems. Through this experience, I have become more mature and have gained a better sense of myself, and therefore, my goals and interests.

Besides of working and participating in the university life I took the advantage of being in North Carolina and tried to explore the beauties of the State. We spent nice weekends on the tracks of the Appalachians. The appeal was great and enduring.

I participated at the local meeting of Fulbrighters in the North Carolina. We spent very good time together in Asheville in the Great Smoky Mountains. Everything was simple but the feeling was great. I have gotten to know many people from all over the world and received a rare insight into their many different cultures and backgrounds. Most importantly, this opportunity has allowed me to experience countless things that I might not have attempted to explore elsewhere.

I have been working as a full professor at my university. Therefore I do not expect formally too much from the Fulbright in promoting my personal career. In fact, I am only just beginning to understand the value of the professional connections that one can make through the Fulbright. The Fulbright has been of major benefit because it's cross-disciplinary perspective. The experiences I gained during the application period, during the introductory training and during my fellowship period and the fact that I now belong to the Fulbright family reaffirmed my trust in the strength of science. I think organizations like the Fulbright can bring people working in the academic field closer and help in finding solutions in tough economic and political situations in the world.

I am thankful to Wake Forest University School of Medicine that allowed me to use the enormous academic potential and the high-tech facilities for studying the mechanism of perinatal hypoxic brain injuries. I think that our joint efforts with Professor Busija and with his coworkers expanded our previous observations and we provided some exciting new discoveries. Since I left the lab in the Wake Forest University my colleagues have been working on the issues I started with. I was happy to realize that the ideas

generated during my short stay in the lab were useful for my young coworkers. Since then we have been communicating almost every day. This seems to me as an extension of my Fulbright period.

3.1. Publications related to my Fulbright Research Award

Nagy K, Kis B, Rajapakse NC, Bari F, Busija DW (2004) Diazoxide preconditioning protects against neuronal cell death by attenuation of oxidative stress upon glutamate stimulation J Neurosci Res 76:697-704

Busija DW, Katakam P, Rajapakse NC, Kis B, Grover G, Domoki F, Bari F (2005) Effects of ATP-Sensitive Potassium Channel Activators Diazoxide and BMS-191095 on Membrane Potential and Reactive Oxygen Species Production in Isolated Piglet Mitochondria Brain Res Bull (in press)

Simandle S, Kerr BA, Lacza Zs, Eckman D, Busija DW, Bari F (2005) Lack of direct dilator effects of N-methyl-D-aspartate on piglet pial arterioles Microvasc Res (in press)

Lenzser G, Kis B, Bari F, Busija DW (2005) Diazoxide preconditioning attenuates global cerebral ischemia-induced blood-brain barrier permeability Brain Res (in press)

Trends in American Luther Research.

Parallels Between Luther's Theology and Shakespeare's Hamlet.

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The research was, first of all, a „metahistorical" investigation into three tendencies of American Luther scholarship: 1. hermeneutics; 2. theology of the cross; and 3. ecclesiology. I was interested to learn how these three tendencies reflected the changing perspective in American Luther Research in the second half of the 20th century.

On the other hand, however, I was interested to demonstrate that Luther's theology can be applied to interpreting Shakespeare's plays. Therefore I was investigating how Luther's understanding of the hidden God can be applied to Shakespearean tragedy, especially Hamlet Prince of Denmark who was also student of Wittenberg. Luther, who called himself "God's court-jester" (Hofnarr) saw history as one of the "masks of God" (larva dei) and God as hiding himself often in the mask of the Devil, developed a paradoxical theology (theologia crucis) that is, according to the paper, surprisingly compatible with the paradoxical artistic vision of Shakespeare, especially in Hamlet, King Lear and Measure for Measure. In discussing central motifs of Luther's theology like deus absconditus; indirect revelation; revelation by concealment; revelation under the opposite (sub contrario suo); the "strange acts of God" (opus alienum), God's "rearward parts" (posteriora); suffering (Anfechtungen and melancholy) we may provoke the latent, even if blasphemeous, theological meaning in Shakespeare.