POLISH ACADEMY OF SCIENCES
MEDICAL RESEARCH CENTRE

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CONTINUATION OF STUDIES ON THE MECHANISMS AND FUNCTION OF THE CAROTID BODY

Project leader: Mieczysław Pokorski
Research team: Lidia Faff, Robert Strosznajder, Magdalena Marczak, Aneta Dymecka, Ahlam Ramadan, Lidia Zarębska, Jacek Kurnicki

The redistribution pattern of calcium ions in carotid chemoreceptor cells in response to the hypoxic stimulus

In this study, performed on cats, we used the oxalate-pyroantimonate technique that yields an electron-opaque calcium precipitate in the cell. The major finding of the study was that in hypoxia calcium precipitates shifted from organelles to the cytoplasm. These results indicate the involvement of subcellular calcium trafficking in hypoxia-sensing in the carotid body.

The redox signaling in carotid body function

The aim of this study was to find out whether vitamin C supplemented in the form of the lipophilic ester ascorbyl-6-palmitate could influence the carotid body function, as measured from the neural respiratory response to hypoxia. The idea was that ascorbyl palmitate could act as a carrier of the ascorbate moiety into biomembranes.

We found that ascorbyl palmitate is able to penetrate both central and peripheral neural tissues. It accumulates vividly in the cat carotid body. It also has physiological activity with respect to respiration, which consists of enhancing the responses to hypoxia in the cat preparation.

Inhibitory component of carotid body function

A series of studies has addressed the issue of the existence of an inhibitory component in normal carotid body function. Excitation of the carotid body usually stimulates respiration. Some data in the literature indicated, however, the organ may have also an inhibitory influence on respiration, which comes to light mostly during protracted exposure to hypoxia. We hypothesized that such an inhibitory part, if present at all, could also be expressed during the stimulatory phase mediated by the carotid body. We used a period of pure oxygen breathing as tool to expose the inhibitory component by blocking the stimulatory one. The results indicate that the hypoxic response is augmented after the oxygen breathing. The magnitude of this augmentation may be taken as measure of the inhibitory component that under normal conditions mitigates the carotid body function and thus also ventilation. This has been a human study that will be continued in the coming year.

Poly(ADP-ribose) polymerase in the carotid body

A biochemical study compared the activity of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme, in the hypoxic and hyperoxic cat carotid bodies, two extreme toxic stimuli. Unexpectedly, it has been found the both stimuli increase this activity. The underlying background of these effects could be the free radical reactions taking place due to both contrasting stimuli. The results suggest that PARP might be involved in protecting nuclear DNA in chemoreceptor cells when toxic stimuli are at work, regardless of their nature. Therefore, not that much the kind of stimulus matters as the nuclear events evoked by it.

A series of ultrastructural studies has been carried out, which concerns the effects of aging on carotid body parenchyma. The study compared the subcellular components of carotid body chemoreceptor cells excised from young (3 mo) and old (2 yr) rats. We found a varied degree of degenerative changes, especially localized to chemoreceptor cells. Among them, there were prominent changes in mitochondria consisting of blurred cristae, vacuolization or their decomposition. Such changes might explain blunting of the respiratory response to hypoxia observed in some studies with age. The study will be continued.

EFFECT OF AMYLOID PEPTIDE AND OTHER PROOXIDANTS ON ARACHIDONIC ACID AND PHOSPHOLIPID METABOLISM IN PERICYTES

Project co-ordinator: Robert Strosznajder

The inhibitory effect of amyloid beta 1-42 and its 25-35 fragment on phosphatidylcholine metabolism in pericytes was investigated in collaboration with Prof. Alberghina, University of Catania, Italy. Treatment of pericytes with 50 μM AB 1-42 or 25-35 for 24 h significantly increased either ^{3}H-choline or ^{14}C-arachidonic acid release. Concomitantly, both fragments of amyloid beta significantly decreased ^{3}H-choline incorporation into PtdCho. This inhibitory action suggests that the amyloid beta protein may modulate pericytes phospholipid synthesis and may contribute to the cerebrovascular degeneration in Alzheimer's disease and related disorders.

In the experiments with oxidized low density lipoprotein (oxLDL) and tertbutyl hydroperoxide (t-BuOOH) the prooxidant signals were tested in retinal pericytes, previously activated by lipopolysaccharide (LPS). t-BuOOH (200 μM) and oxLDL (100 μM) but not native LDL markedly increased lipid peroxidation, cytosolic phospholipase A2 activity and arachidonic acid release. t-BuOOH and oxLDL applied together synergistically increased phospholipid hydrolysis induced by the LPS alone. These results clearly show that pericytes

may be the target of extensive oxidative damage and indicate that *in vitro* activation of pericytes may serve as a model for the study of inflammation on the blood-brain or blood-retina barrier.

EXCITATORY AND INHIBITORY NEUROTRANSMITTERS IN CENTRAL AND PERIPHERAL REGULATION OF BREATHING

Project leader: Krystyna Budzińska
Contributors: Krystyna Budzińska, Beata Sokołowska

Neurophysiological studies were conducted on the role of GABA-ergic and glycinergic neurotransmission in generation of apneustic breathing. The apneustic breathing (pronounced prolongation of inspiration) is a sign of an impairment of mechanisms switching inspiration to expiration. NMDA antagonists like ketamine evoke apneustic pattern of breathing supporting the idea that activation of NMDA receptors is involved in the central mechanism of inspiratory termination. Besides, inhibitory processes transmitted by $GABA_A$ and glycine receptors play an important role in respiratory rhythmicity. Since ketamine increases GABA level in some brain structures and increases GABA-dependent chloride current we hypothesized that the mechanism of NMDA-mediated apneustic breathing may also include a component involving inhibitory amino acids. We found that non-competitive $GABA_A$ antagonist, picrotoxin acts antagonistically to apneustic effects of ketamine, mostly by a reduction of respiratory depression and to a lesser degree by excitation of a phase transition. Bicuculline, a competitive $GABA_A$ antagonist decreases duration of apneustic inspiration and maintains respiratory depression. Glycine antagonist, strychnine, is only slightly agonistic to ketamine, in terms of inspiratory duration. The results indicate the differential modulatory role of $GABA_A$ and glycine antagonists on apneustic respiration evoked by NMDA receptor antagonist ketamine. Excitation and inhibition in the respiratory network, mediated respectively by ionotropic glutaminergic, GABA-ergic and glycinergic receptors, consists of tonic and pha-

sic components. NMDA receptors are responsible for tonic while non-NMDA receptors for phasic excitation. Similarly, $GABA_A$ antagonists exert divergent effects on tonic and phasic neuronal activities. Since both NMDA receptors and $GABA_A$ antagonist, picrotoxin affect tonic respiratory activities, antagonistic effects of picrotoxin and ketamine on apneustic breathing probably originate from their interaction on tonic excitation and inhibition of the respiratory neurons.

We continued studies on diagnosis of the respiratory dysfunction by methods of statistical pattern recognition on an experimental model of diaphragm denervation. The previous data analysis was performed with the use of the standard *k* nearest neighbour (*k*-NN) rule. At present, two new more sophisticated methods of pattern recognition were being: a fuzzy version of the *k*-NN rule and a multistage classifier. The misclassification rate was the unique criterion for evaluation as the standard classifier as well as its fuzzy version. This rate is the only indicator of the classification confidence and is associated with the set of cases. In comparison with the standard *k*-NN rule, the fuzzy *k*-NN classifier used in our recent studies offered significantly smaller misclassification rate.

However, we feel that classification of some cases may be very clear while other cases are more difficult to be recognized. We are interested to use such a method that would enable to evaluate whether the assigned class is almost sure or only the most probable. This property possesses newly developed multistage classifier, which we have also applied for the data analysis. Both ideas, the one based on a fuzzy approach and the second that uses a multistage scheme can be combined.

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Publications

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STUDIES ON THE CONTRIBUTION OF SELECTED NEUROTRANSMITTERS TO LUNG REFLEXES

Project leader: Małgorzata Szereda-Przestaszewska
Contributors: Katarzyna Kaczyńska, Beata Kopczyńska

Gamma amino-butyric acid (GABA) is the most important inhibitory neurotransmitter in the vertebrate CNS. GABA was also identified in the peripheral nervous system, in airways' nonmyelinated C fibres. Since GABA does not readily cross the blood-brain barrier, studies using its systemic administration can be regarded as a search for peripheral effects.

In this study intracarotid injections of GABA in either intact or midcervically vagotomized rats evoked apnoea, followed by stimulated breathing, due to the action on the tidal component of the breathing pattern. Blockade of $GABA_A$ receptors wild bicuculline and picrotoxin abolished the inhibition of breathing. Sections of the carotid sinus nerves in vagotomized rats precluded the occurrence of apnoea and respiratory changes evoked by GABA.

The results suggest that GABA given systematically induced depression of respiration, followed by stimulation, occurring beyond the vagal loop and abolished by interruption of the carotid body input.

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Publication

Szereda-Przestaszewska M, Kopczyńska B, Kaczyńska K, Chrapusta SJ: Diverging respiratory effects of serotonin and nicotine in vagotomized cats prior and after section of the carotid sinus nerves. *J Physiol Pharmacol* 2001, 52, 71-79.

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PHARMACOLOGICAL NEUROPROTECTION

Project leader: Paweł Grieb
Contributors: Mirosław Ryba, Bronisław Głód, Stanisław Chrapusta, Ewa Wojtal, Tomasz Kryczka

Properties of a neuroprotective drug citicoline (CDP-choline) have been further characterized. It has been found that repeated treatment of rats with citicoline leads to a significant increase in dopamine content in retina. This effect may contribute to the beneficial effects of citicoline in glaucoma.

Liposomes containing lipophilic antioxidant coenzyme Q10 have been tested for neuroprotective properties using gerbil forebrain ischemia model and murine pharmacologically induced lethal seizures model. No evidence of neuroprotection has been found.

Chromatographic assay of the products of hydroxyl radicals quenching by a non-toxic spin trapping agent p-hydroxybenzoic acid has been developed. This methodology may enable to quantify the rate of hydroxyl radicals production *in situ*.

Preliminary data concerning immunohistochemical localization of two isoenzymes of kynurenine aminotransferase (KAT1 and KAT2) in the gerbil brain have been obtained with the use of specific antibodies.

CDP-CHOLINE, BUT NOT CYTIDINE, PROTECTS HIPPOCAMPAL CA1 NEURONS IN THE GERBIL FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA

Project leader: Paweł Grieb
Contributors: Roman Gadamski*, Renata Wojda*, Monika Janisz.

The effects of CDP-choline (cyticoline), cytidine monophosphate or cytidine on the number of CA1 hippocampal neurons surviving five-minute forebrain ischemia have been evaluated in gerbils. The substances tested were given in daily doses, equivalent on a molar basis to 500 mg/kg CDP-choline, starting immediately after ischemia. On day five, the brains were perfused, postfixed, cut into 10 μm slices and stained with cresyl violet, and the number of neurons in the CA1 sectors was counted manually under a light microscope at magnification x 400. The results indicate a significant degree of protection provided by cyticoline, but no protection by cytidine monophosphate or cytidine. The choline moiety of CDP-choline appears to be essential for the neuroprotective properties of the drug.

BRAIN GLUCODEPRIVATION AS A POTENTIAL ANIMAL MODEL OF ALZHEIMER'S DEMENTIA

Supported by the State Committee for Scientific Research: grant # 4P05 A04117

Project leader: Mirosław J. Mossakowski*
Contributors: Paweł Grieb, Tomasz Kryczka, Wanda Gordon-Krajcer**

Experiments on rats have been conducted to compare the effects of acute brain glucodeprivation by subtoxic doses of a nonmetabolyzeable glucose derivative 2-deoxyglucose (2-DG) and chronic glucodeprivation by intracerebroventricular injection of streptozoto-

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cin (STZ) believed to damage insulin receptors on beta-APP expression and tau protein phosphorylation.

A methodology enabling *in vivo* recordings of brain proton and phosphorous magnetic resonance spectra and their changes following glucodeprivation was developed.

BIODEGRADABLE POLYMERS CONTAINING NUCLEOSIDE ANALOGS FOR THE TREATMENT OF BRAIN TUMORS

Supported by the State Committee for Scientific Research: grant # 405F 02412

Project leader: Paweł Grieb
Contributor: Tomasz Kryczka

Intracranial implantation of biodegradable polymers containing cytotoxic drugs is a relatively new and promising technique of brain tumor chemiotherapy. One of the major problems in the development of clinically applicable polymeric implants concerns their sterilization, which may change polymer structure and kinetics of release of cytotoxic compounds. The effects of ethylene oxide and gamma-ray sterilization on the kinetics of release of a cytotoxic nucleoside cladribine from a series of biodegradable lactide-glycolide or lactide-caprolactone copolymers have been assessed with the use of the *in vitro* model developed previously. The results obtained are suggestive of that the sterilization by radiation shall be preferred, because it only slightly influences the kinetics of the cytotoxic nucleoside release from the copolymers.

IN VITRO MAGNETIC RESONANCE SPECTROSCOPY OF BRAIN TUMOR TISSUES – APPLICATIONS IN CLINICAL DIAGNOSTICS OF TUMORS

Supported by the State Committee for Scientific Research: grant # 405F 02412

Project leader: Zbigniew Czernicki (Department of Neurosurgery)
Contributor: Paweł Grieb

The database of proton magnetic resonance spectra of water-soluble extracts of brain tumor tissues has been expanded, and various statistical approaches have been applied in order to classify tumors on the basis of their MR "fingerprints". Furthermore, the High Resolution Magic Angle Spinning (HR-MAS) technique has been applied for the first time for the spectral analysis of lyophilized brain tumor tissue speciments. High-quality proton HR-MAS-MRS spectra have been obtained, but it is difficult to asses at this stage whether they contain clinically useful information related to tumor histology.

Collaborating units

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Publications

Grieb P, Gadamski R, Wojda R, Janisz M: CDP-choline, but not cytidine, protects hippocampal CA1 neurons in the gerbil following transient forebrain ischaemia. *Folia Neuropathol* 2001, 39, 141-145.

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NEURONAL PLASTICITY, NEURODEGENERATION AND NEUROPROTECTION IN CNS: ROLE OF GLUTAMATE RECEPTORS AND CALCIUM IONS

Project leader: Jerzy W. Łazarewicz
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Effects of NMDA receptor antagonist on the induction of a long-term ischemic tolerance and on a rapid anoxic preconditioning were studied *in vivo* in the brain of Mongolian gerbils and *in vitro* in rat cortical slices, respectively. *In vivo* normothermic model of 2-min ischemic preconditioning (2 vessel occlusion), followed after 48 h by 3-min test ischemia, demonstrated a significant reduction of neuronal loss in the CA1 sector of the hippocampus, from 74.6% to 19.6%. This phenomenon was not affected by memantine, an uncompetitive NMDA receptor antagonist with low affinity to the NMDA channel, when the drug was administered i.p. 1 h before preconditioning in a dose of 5 mg/kg. A chronic memantine infusion (30 mg/kg, s.c.) for 3 days before test ischemia provided significant neuroprotection when given to non-preconditioned and particularly to preconditioned

rats (neuronal loss 49.1% and 6.4%, respectively). In the model of a rapid anoxic preconditioning of the rat cortical slices, 10-min test anoxia was preceded for 90 min by 2-min anoxia, and calcium transients were evaluated fluorimetrically. After preconditioning anoxia, a mild increase in the levels of intracellular free and bound calcium was observed, interpreted as enhanced calcium signalling. Test anoxia resulted in a massive accumulation of calcium, reflecting disruption of calcium homeostasis. Preconditioning prevented development of these pathological calcium transients, whereas high affinity uncompetitive NMDA receptor antagonists 10 μM MK-801 or a competitive antagonist 30 μM APV applied during preconditioning, reduced anoxic tolerance. These data indicate that NMDA receptors may be involved in the induction of neuronal tolerance to ischemia/anoxia. They visualise differential effects of high and low affinity NMDA channel blockers. MK-801, which strongly blocks the channel, inhibits its induction of tolerance, whereas a low affinity antagonist memantine induces neuroprotection and potentiates induced tolerance to ischemia.

Utilising the gerbil model of forebrain ischemia, the phosphorylation of tau protein was examined in homogenates of the gerbil brain cortex during 5 min ischemia and reperfusion. Homogenates were analysed by Western blotting. Using antibodies against non-phosphorylated (Alz 50, Tau 14 and Tau 46) tau epitopes, the 63, 65 and 68 kD polypeptides were detected in control animals, whereas antibodies 12E8 and PHF-1 against phosphorylated epitopes were immunoreactive with only a single but dissimilar polypeptides of 63 kD and 68 kD, respectively. Ischemia resulted in a significant decrease in the PHF-1 immunoreactivity, which recovered during 20 min of recirculation and increased above control level 3 and 7 days later. In contrast, the 12E8 immunoreactivity increased during ischemia and was still partially elevated at 2 h of recirculation. The results may suggest differential mechanisms regulating phosphorylation/dephosphorylation of these sites and their different roles in microtubule dynamics. These sites may originate from various pools of vulnerable neurons or glial cells in the brain.

It has been recently suggested that dysfunction of endoplasmic reticulum (ER), triggered by depletion of ER calcium pool, may result in neurodegeneration in various pathological conditions including brain ischemia. Addressing this subject, studies were initiated on the effect of brain ischemia on the NMDA-evoked Ca^{2+}-induced Ca^{2+} release (CICR) *via* ryanodine receptors (RyR). CICR was detected as an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in synaptoneurosomes isolated from the rat brain cortex and hippocampus, one, two and three days after 10-min forebrain ischemia (4 vessel occlusion induced according to Pulsinelli). Isolated crude synaptoneurosomal fractions loaded with fura-2 were stimulated with 0.5 mM NMDA, and the effects of RyR blockers, ryanodine or dantrolene was detected. Our results demonstrate that in synaptoneurosomes isolated from the hippocampus, but not from the cortex, NMDA-evoked increase in $[Ca^{2+}]_i$ on the first and second day after ischemia is attenuated in about 30%, and on the first day its ryanodine- and dantrolene-sensitive portion disappeared. On the third day after ischemia, NMDA-induced increase in $[Ca^{2+}]_i$ was even potentiated, and its ryanodine- and dantrolene-sensitive portion partially recovered. Thus, 10-min forebrain ischemia in rats induces transient inhibition of the NMDA-evoked CICR in the hippocampal neurons.

Collaborating units

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EFFECT OF METABOTROPIC GLUTAMATE RECEPTOR AGONISTS ON PROCESSES OF LEARNING AND MEMORY FORMATION INDUCED BY PASSIVE AVOIDANCE TRAINING IN A ONE-DAY-OLD CHICK

Supported by the State Committee for Scientific Research: grant # 4 P05A 111 19

Project leader: Elżbieta Salińska

The aim of this study is the detection of the involvement of metabotropic glutamate receptors in processes connected with learning and memory and establishing its mechanisms. Effects of intracerebral injections of glutamate metabotropic receptor agonists ACPB and ABHxD-I on memory consolidation was studied in a one-day-old chicks. A dose-effect relation was detected and a minimal active dose (25 nmoles per hemisphere) was established for both agonists. It was found that application of this dose in the period of 1 h around the passive avoidance training induced amnesia in chicks. The strongest amnestic effect was detected in the animals which were treated with agonists 30 min before the training and tested 30 min and 3 h after that. Amnestic effect of ABHxD-I was stronger and lasted longer than after ACPD application. Effect of agonists of glutamate metabotropic receptors on glutamate release *ex vivo* was studied. Significantly higher release was found in brain slices of trained chicks. Injections of ACPD and ABHxD-I slightly potentiated glutamate release in control chicks, but had no effect on glutamate release in trained animals.

Collaborating unit

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ROLE OF RYANODINE-SENSITIVE INTRACELLULAR CALCIUM STORES IN MITOCHONDRIAL PERMEABILITY TRANSITION IN RAT CEREBELLAR GRANULE CELLS

Supported by the State Committee for Scientific Research: grant # 4 P05A 031 17

Project leader: Elżbieta Ziemińska

Relation was studied between hypothetical mechanisms of neurodegeneration: calcium release from endoplasmic reticulum (ER) *via* ryanodine receptors (RyR) on one hand, and mitochondrial calcium overload, their permeability transition (MPT), swelling, and release of the proapoptotic cytochrome c, on the other. Cultured cerebellar granule cells were incubated for 30 min in the presence of glutamate (0.1-1 mM). Ultrastructural changes of mitochondria and cytochrome c release were determined immediately after incubation. Neuronal death was assessed 24 h later, by measuring LDH release to the medium. It was found that incubation with 0.5 μM cyclosporin A (CsA), a blocker of MPT, and also with 30 μM dantrolene, RyR antagonist and stabiliser of intracellular calcium, partially prevents ultrastructural changes of mitochondria, reduces cytochrome c release and protects neurons from degeneration. Neuroprotective potential of CsA was higher than of dantrolene. Thus, dantrolene-evoked stabilisation of intracellular calcium homeostasis may partially prevent MPT under conditions of acute excitotoxicity.

ROLE OF HYPERHOMOCYSTEINEMIA IN PATHOGENESIS OF ISCHEMIC NEURONAL INJURY

Supported by the State Committee for Scientific Research:
grant # K 018/P05/2001 task # 6, "Molecular and genetic mechanisms of neurodegeneration and neuroprotection"

Project leader: Jerzy W. Łazarewicz

At the end of 2001, a 2-year pre-clinical and clinical study was initiated on possible direct participation of homocysteine in the mechanisms of neuronal damage in stroke. Clinical material is being collected. *In vitro* and *in vivo* experiments were initiated aimed at the detection of the mechanisms of homocysteine neurotoxicity, particularly connected with excitotoxicity and oxidative stress. Studies are in progress.

Collaborating unit

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MECHANISMS OF DANTROLENE - EVOKED NEUROPROTECTION IN MODEL PERINATAL ASPHYXIA IN RATS

Supported by the State Committee for Scientific Research:
pre-doctoral grant # 6 P05A 017 21

Project leader (supervisor of the thesis): Jerzy W. Łazarewicz
Contributor (Ph.D. student): Dorota Makarewicz

In the previous recently completed project, a neuroprotective potential of dantrolene, antagonist of the ryanodine channels and inhibitor of intracellular calcium mobilisation was demonstrated in a model of perinatal asphyxia (hypoxia/ischemia of neonatal rats). In these studies, systemic administration of dantrolene 30 min after the insult significantly reduced brain damage without influencing body temperature. Studies initiated at the end of 2001 are aimed at the

explanation of the mechanism of dantrolene neuroprotection in the model of perinatal asphyxia. Experimental material is being collected for studies on the relation between localisation of ryanodine receptors (RyR) and dantrolene-induced neuroprotection in different brain regions. Other experiments concern expression of [^{3}H]ryanodine binding sites and of RyR isoforms in developing rat brain. Studies are in progress.

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THE STUDIES ON THE DISTURBANCES OF METABOLISM AND FUNCTIONS OF BRAIN SUBSTRUCTURES CAUSED BY ISCHEMIC AND POSTISCHEMIC PATHOLOGY AND TOXICITY OF LEAD

Project leader: Urszula Rafałowska
Contributors: Lidia Strużyńska, Grzegorz Sulkowski, Jolanta Waśkiewicz, Aleksandra Lenkiewicz

Alteration of GABA transport in brain induced by global ischemia caused by cardiac arrest and reperfusion in rat

This study was designed to determine the effects of global cerebral ischemia caused by temporary cardiac arrest and the early and late consequences of this ischemia and reperfusion on GABA transport and $GABA_B$ receptor binding in rat brain.

The effects of 10 min of global ischemia were measured instantly and at 1h, 24 h and 7 days post resuscitation. Global ischemia caused drastic decrease in GABA uptake and release. This effect was slightly enhanced at 1h and 24 h post resuscitation. The uptake and release of GABA in synaptosomes normalized at 7 days post resuscitation. Global ischemia and reperfusion also affected $GABA_B$ receptor binding, increasing its affinity (reduced K_D) and decreasing the density of the receptor (B_{max}). The kinetic parameters of $GABA_B$ receptor binding normalized after 7 days post resuscitation.

Glutathione and glutathione-related enzymes in rat brain after acute lead exposure

Numerous studies confirm the adverse effects of lead (Pb) on CNS function. Since glutathione is cytoprotective against oxidative damage exerted by endo- and exogenous toxins, we studied the effect of lead on the homeostasis of subcellular GSH system. The total glutathione level and the activities of two enzymes involved in its metabolism i.e. γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme for GSH *de novo* synthesis, and glutathione reductase (GR), involved in the regeneration of GSH from its oxidized form were determined. Adult rats were injected i.p. with 25 mg of lead acetate/kg b.w. for 3 days. Total glutathione level was significantly elevated when measured in the brain fraction highly enriched in mitochondria. The activities of both enzymes, γ-GCS and GR, were increased in the mitochondrial fraction by about 50% and 70%, respectively. The activities of enzymes measured in the cytosolic fraction were unchanged. The results indicate an active response of the antioxidant GSH system in the mitochondrial compartment of the adult rat brain to Pb.

Publications

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SENSORING AND SIGNALLING PROTEINS IN APOPTOSIS INDUCTION AFTER CEREBRAL ISCHEMIA

Project leader: Krystyna Domańska-Janik
Contributors: Agnieszka Bronisz, Leonora Bużańska, Joanna Sypecka, Anna Sarnowska

Intracellular signals responsible for delayed ischemic death or survival are generated immediately during and after the insult and further propagated by a variety of kinases, proteases and phosphatases. Still poorly defined factors determining local susceptibility of certain brain regions or cells such as pyramidal neurons of CA1 hippocampus, to ischemic injury may rely on spatial differentiation of these postischemic signalling. Tissue samples from dorsal (vulnerable) and abdominal (resistant) parts of gerbil hippocampus were collected to determine the activation state of several key signalling molecules: Akt, Raf-1, JNK, ERK1/2 in the time course of reperfusion after 5 min of global cerebral ischemia. Western blot analysis of phosphorylated/activated forms of these kinases revealed persistent activation of JNK, mostly limited to the vulnerable CA1 region. On the contrary, activation of ERK, although observed transiently in both parts, was enhanced for a longer time in the abdominal hippocampus. The levels of active/phosphorylated Akt and Raf-1 kinases did not change significantly during the entire recovery period. We have found a rather poor correlation between postischemic JNK activation and c-Jun phosphorylation or its contribution to AP1-like complex formation. In contrast, the amount of active/phosphorylated JNK connected with mitochondrial membranes was significantly increased

at the time directly preceding neuronal death. It may be of interest that at the same time the AP1 complex, augmented in CA1 region, did not appear to contain a classical c-Fos protein. These results, together with other previous studies indicate that, either long-lasting activation of JNK or discrepancy between ERK and JNK activation in critical time of reperfusion, contributes to selective apoptosis of CA1 neurons. This, in connection with translocation of active JNK toward mitochondria and regional differences in AP1 binding protein complexes, the nature of which remains to be determined, can modulate the final postischemic outcome.

Recent findings support the hypothesis that mitochondria and the mtPTP (mitochondrial permeability transition pore) play a pivotal role in determining the destiny of cells after various lethal injuries. During reperfusion after transient cerebral ischemia, elevated intracellular calcium is taken up by re-energized mitochondria which are prone to production of reactive oxygen species. These early events promote reversible, temporal opening of a large pore on the mitochondrial inner membranes (mtPTP). Later on, depending on the restoration of energy metabolism during reperfusion, these early changes can be, slowly but steadily, normalized. In selected cell populations or subcellular compartments however, a more severe disruption of the mitochondrial function could be irreversible. This results in their further osmotic swelling which, concomitantly with other pathological signals produced by postischemic dysfunctions of signalling cascades, can contribute to leakage of pro-apoptotic factors from mitochondria and delayed death of neurons in selected brain areas.

Cyclosporine A (CsA) is the compound which besides its well known immunosuppressive activity can stabilize mitochondrial membrane and block mtPTP both *in vitro* and *in vivo*.

In agreement with other data we have demonstrated that CsA can protect CA1 neurons of hippocampus in the model of 5 min. cerebral ischemia in gerbils. We have shown that a single, intercarotid injection of CsA in a dose of 5-10 mg/kg given immediately after the insult significantly increases the average number of the survived pyramidal neurons. There was no protection when CsA was

applied after 6 to 48 hr recirculation – the time when neurogenic inflammation starts to contribute to postischemic brain pathology.

To get more insight into the mechanism of CsA protection, we have performed experiments on mixed, primary cortical cultures *in vitro*. By differential cell staining with Hoechst 33258 and immunochemical markers of neurons and glia we have shown that apoptotic response can be minimized in the presence of CsA in the medium. This neuroprotective effect of CsA, similar to that observed *in vivo*, was time-dependent and disappeared at 3 h post treatment. In conclusion, our results confirm therapeutic potential of CsA in global cerebral ischemia *in vivo* as well as in several models of neuronal apoptosis *in vitro*, but only when the drug has been applied within a relatively short time after the insult.

In an other study (doctor thesis of A. Bronisz), neuroblastoma N2a cell line was applied to investigate mechanisms of neuronal death and survival. Biochemical pathways leading to apoptosis were studied in the models of staurosporine- and wortmanin-induced cell death. We have verified the hypothesis that Raf 1, which is activated by dual action of Ras and PKC kinases can, together with PIP3K/Akt pathway, phosphorylate Bad and then, due to enhanced cytoplasmic sequestration of P-Bad protein, inhibits its proapoptotic function and promotes cell survival. N2a cells were treated by a low,10 nM concentration of staurosporine (STS10) shown to inhibit selectively PKC activity. Decreased phosphorylation of Raf 1 and Bad (on 112 serine residue) proteins was observed at 6 hrs after induction of apoptosis. Concomitantly, release of cytochrome C into cytosol and translocation of Bad to mitochondria were found by Western blot and immunoelectron microscopy analysis, respectively. Furthermore, cells with over-expression of mitochondria-tagged, permanently active Raf 1 transgen displayed significantly higher resistance to STS10 but – not to WM – induced apoptosis. In contrast, application of WM (300 nM) resulted in a substantial reduction of Akt but not Raf 1 phosphorylation. This treatment, similarly to STS10, evoked a mitochondria-dependent apoptotic response with dephosphorylation of Bad (but in this case on 136 serine residue) and release of cytochrome C to

cytosol. In parallel, N2a cells with overproduction of permanently active Akt kinase construct were resistant to WM but not to STS10 treatment. In addition, although apoptosis induced by STS10 and WM seems to underly two different, independent pathways, the effects of the particular inhibitors on N2a cell survival were not additive and in each case caused about 30% of cell death after 6 h post-treatment. This is in agreement with the assumption that two independent pro-survival signalling pathways involving Raf1 or Akt kinases converge at the common level of BAD phosphorylation at the different serine (112 or 136) residues. These pathways are independently induced by PKC and IP3K activity, respectively, and both are necessary for cell survival due to dual phosphorylation-dependent sequestration of proapoptotic BAD protein. Importantly, each apoptotic response was blocked by the presence of serum in growing medium; however this protection was abrogated when STS10 and WM were applied simultaneously, as well as after treatment by an unspecifically high, micromolar staurosporine concentration. Thus, our results indicate at either of the two pathways: PKC/Raf 1/Bad and PIP3K/Akt/Bad is critical for cell survival and none is able to substitute functionally the other as long as the cells do not receive additional signal(s) derived from serum. This undefined signal(s) would stimulate the cross-talk between these two pathways at the level upstream from Raf 1 and Akt phosphorylation. Therefore, only simultaneous inhibition of PKC and PI3K is able to induce apoptosis in the presence of serum. On the contrary, in the absence of serum-derived trophic support, inhibition of only one of these two initiating kinases would lead to cell death. This mechanism can operate during differential modulation of growth factor regulated neuronal survival in developing or injured brain.

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HUMAN, UMBILICAL CORD-DERIVED STEM CELLS AS A SOURCE OF NEURONS, ASTROCYTES AND OLIGODENDROCYTES FOR POTENTIAL THERAPEUTICAL APPLICATIONS

Project leader: Krystyna Domańska-Janik
Contributor: Leonora Bużańska

In this study we have provided evidence, to our knowledge for the first time, that a subfraction of human cord blood mononuclear (CB) cells can give rise to all three types of brain cell phenotypes – neurons, astrocytes and oligodendrocytes. In parallel, Sanches-Ramos et al. (*Exp. Neurol.* 2001, 171, 109-115) reported similar finding; however in this study neural differentiation has been limited only to neurons and astrocytes.

Briefly: the mononuclear fraction was purified on Ficoll gradient and immunodepleted of CD34+ cells by the antibody-driven, magnetic sorting. Cells were selected further during cultivation in the defined, "discriminative" conditions *in vitro*. FACS analysis as well as a hematopoietic-colony-formation-test revealed selection of CD34-, CD45- cell population with a relatively high expansion potential and with the ability to grow nestin-expressing clones with typical neural precursor properties. In the following study, we have estimated the expression of typical marker proteins for neural (nestin), neuronal (α-tubulin III), astrocytic (GFAP) and oligodendrocytic (GalC and PLP/DM20) differentiation either by PCR, or by immunocytochemical double-labeling or Western blotting. Taking advantage that primary cortical culture can be treated as an alternative to *in vivo* transplantation study, we have shown that CB-derived cells are able to differentiate toward all three types of neural progeny under trophic, paracrine support derived from surrounding brain cells. Moreover, we have evaluated quantitatively the influence of selected mitogens and growth factors on CB-derived cells differentiation *in vitro*, which resulted in maximally about 30% to 40% of cells with neuronal and astrocytic and 11% cells with oligodendrocytic phenotypes.

This study has been supported partially by the State Committee for Scientific Research: grant # 6 P05A 049 20 to Leonora Bużańska.

Collaborating units

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THE ROLE OF PROTEOLYSIS IN NEURODEGENERATION

Project leader: Teresa Zalewska
Contributors: Krystyna Domańska-Janik, Małgorzata Ziemka-Nałęcz, Anna Sarnowska

Previous studies have shown that a temporal and spatial relationship exists between activation of metalloproteinase-9 and neuronal damage following short-term cerebral ischemia: the damage occurred selectively in pyramidal neurons of CA1 region. Continuing these studies we found that the enhancement of proteolytic activity is accompanied by degradation of laminin, a component of extracellular matrix and substrate for metalloproteinase-9. Proteolysis of laminin and progressive accumulation of several intermediate degradation products in hippocampus precedes neuronal cells loss. These data are indicative of disruption of adhesive interaction between neurons and extracellular matrix and dearrangement of the downstream signalling pathways. It is suggested that one of the early steps in a signal transduction cascade that permits the flow of information from extracellular compartment to interior of the cell is non-receptor focal adhesion kinase - pp125FAK. Therefore in the further study we have explored the involvement of ECM-pp125FAK signalling pathways in ischemia-induced neuronal degeneration. We estimated the level of total FAK as well as its phosphorylated form, which can serve as an index of kinase activity. We have found that ischemia resulted in proteolytic cleavage of total FAK, which coincides temporarily with proteolytic modification of extracellular matrix components. Furthermore, we have observed an early post-ischemic enhancement of

FAK phosphorylation/activation, followed by its marked decrease at a later time. We conclude, that ischemia can influence the pp125FAH activity directly, which together with proteolytic degradation of extracellular matrix and ensuing deregulation of this signalling pathway, can contribute to delayed neuronal death.

In addition the laboratory participated in a collaborative study concerning the involvement of metalloproteinases in neonatal hypoxic-ischemic brain injury. The results demonstrate similar developmental expression pattern of investigated metalloproteinases in the brain of immature rats: the highest level of activity is seen in the brain of 7 days old pups. Between 7-14 days there is a tendency to a small but significant decrease. A marked decline is observed at 21 days. It was found that ischemia induces more significant activation of extracellular proteinases in the ischemic hemisphere than in the contralateral one. Simultaneously the level of pp125FAK as well as degree of its phosphorylation decreased significantly in the ischemic hemisphere.

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CHANGES IN THE EXPRESSION OF PROTEINS AND GENES IN THE PATHOLOGY OF BRAIN ISCHEMIA

Project leader: Barbara Zabłocka
Contributor: Joanna Dłużniewska

Animal models of transient brain ischemia provide an opportunity to study biochemical pathways leading to delayed neuronal death in vulnerable region (hippocampal CA1) and to survival in the resistant areas. After transient cerebral ischemia several signals involved in initiation of apoptosis seem to integrate on outer mitochondrial membranes resulting in execution of cells death. The aim of this study was to investigate the influence of signal transduction proteins on the make up of mitochondrial membrane in the time course of postischemic reperfusion. In the model of 5 min global brain ischemia in gerbils, mitochondria from hippocampi were analyzed in two periods after the insult: up to three hours of reperfusion, when possible protection of neurons can be provided and in time longer than 24 hours of reperfusion. In both postischemic periods, protein kinases connected with signal transduction seem to influence the permeability of mitochondrial membranes for cytochrom c. In the short reperfusion time, the decrease of death-connected P-JNK kinase was observed. After 24 hours, the amount of phospho-JNK protein connected with mitochondria increased steadily and reached almost 250 % of control value at 72 hrs postischemia. The mitochondrial target proteins for this kinase are still unknown, but *in vitro* data suggest their involvement in the regulation of mitochondrial outer membrane permeabilization connected with the changes in the amount of

phospho-Bcl_{xl} and Bcl2 proteins. Concomitantly, the amount of phospho-Raf-1 kinase, which has been shown to exert anti-apoptotic effect on mitochondria, decreases in mitochondrial fraction at 24 and 48 hrs after ischemia. It corresponds with decreased phosphorylation of one of the Raf-1 kinase target protein, Bad. The changes in the balances between anti- and pro-apoptotic proteins from Bcl2 family may change the permeability of mitochondrial membrane. Additionally, after about 24 hours of reperfusion and later on, cytochrom c shows up in the cytosolic fraction what can be an indicator of mitochondria membrane leakage and the onset of executive phase of postischemic neurodegeneration. In the first hour after ischemia cytochrom c is also seen in the cytosol, but this transient presence is thought to be an inductive not executive in the process of apoptosis, as compare with no other features of cells death observed in the first day after the insult. At the early postischemia, also the amount of pro-apoptotic Bad is elevated concomitantly with phospho-Raf 1 kinase, which probably can overcome it's "bad" action. These data indicate that ischemia-generated pathological signal induces several early and also delayed changes in the compositions of outer mitochondrial membrane which could be decisive for neurons fate.

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Publication

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MOLECULAR MECHANISM OF BRAIN AGING, AMYLOID BETA NEUROTOXICITY AND POSTISCHEMIC ENCEPHALOPATHY

Project leader: Joanna B. Strosznajder
Contributors: Małgorzata Chalimoniuk, Grzegorz Czapski, Henryk Jęśko, Agata Zambrzycka,

The studies carried out in the Department of Cellular Signaling concerned molecular mechanisms of brain aging and amyloid beta neurotoxicity and the alterations of signal transduction during recirculation after ischemia.

Molecular mechanism of brain aging

Age related alteration of nitric oxide synthase(s)

Contributors: Henryk Jęśko, Małgorzata Chalimoniuk, Joanna B. Strosznajder

In our previous work, a significant increase of NO synthesis was found in old Wistar rat brain. The aim of the present study was to determine the expression and activity of particular isoforms of NOS during rat brain aging (at the ages of 4, 14 and 24 months) in brain cortex, cerebellum and hippocampus under specific pathogen free (SPF) environmental conditions. Moreover, the influence of phosphorylation/dephosphorylation processes on NO liberation by NO synthase(s) during aging was investigated. The activity was measured using a radiochemical [^{14}C] arginine-citrulline assay in homogenates and cytosolic fractions. RT-PCR was used for determination of iNOS and nNOS mRNA. Our results indicate the enhancement of

NO release in all investigated old (27 months old)but not adult (4 months old) brain parts. Significantly higher NO synthase activity was found in old cerebellum. There was no gene expression for inducible NOS in both adult and old brains. In the absence of Ca^{2+} or in the presence of N-nitro-L-arginine (NNLA) the activity of NOS was not detectable. An inhibitor of constitutive NOS isoforms, 7-nitroindazole, which preferentially inhibits nNOS, decreased NO production by 60 and 75% in adult and old brains, respectively. However, using RT-PCR lower amounts of nNOS mRNA were detected in the old brain. The ratio of NOS activity to nNOS mRNA was significantly higher in hippocampus and cerebellum of old brain. In the adult brain, protein kinase inhibitors H-7, H-8 and H-9 significantly increased NOS activity while sodium orthovanadate reduced it. There was no influence of kinase/phosphatase inhibitors on NO release in the old brain. Our results indicate that a constitutive isoform of NO synthase, mainly nNOS is responsible for higher NO release in old brain. The data suggest that alterations of nNOS phosphorylation state plays an important role in the age-related increase of brain NO synthesis that may be involved in protein alteration and DNA damage. The down-regulation of nNOS mRNA expression may be an adaptive mechanism that protects the brain against excessive NO release.

Molecular mechanism of Aβ neurotoxicity

Effect of ApoE4 and Aβ on choline acetyltransferase and phospholipase C

Contributors: Agata Zambrzycka, Joanna B. Strosznajder

The effect of apoE4 on choline acetyltransferase (ChAT) and phospholipase C (PLC) in the absence and presence of Aβ peptides was investigated. The protein level and enzyme activities were evaluated. Immunochemical and radiochemical methods for determination of protein level and enzyme activity were used, respectively. Homogenate and synaptic plasma membranes (SPM) were the source of PLC and phosphatidyl[^{3}H]inositol served as an exogenous

substrate. The results indicate that apoE4 decreases but Aβ peptides have no effect on ChAT activity in adult brain. Moreover, this apolipoprotein significantly inhibited phosphatidylinositol-specific phospholipase C (PI-PLC). ApoE4 increased selectively the protein level of PLCδ by 25% but it had no effect on β and γ isoforms. Our data indicate that apoE4 itself inhibits PI-PLC and through the modulation of diacylglycerol level it may influence the signal transduction processes.

Aβ peptides evoked macromolecules oxidation and DNA damage is responsible for nerve endings degeneration and cell death

Contributors: Agata Zambrzycka, Grzegorz Czapski, Joanna B. Strosznajder

In the past year, we have investigated the oxidative damage caused by aggregated Aβ peptides to lipids, proteins and DNA in different brain parts and subcellular fractions.

Our results indicated that lipid peroxidation was increased by both Aβ 1-40 and 1-42 by about 40-50%, but not by Aβ 25-35. However, Aβ 25-35 enhanced conjugated double bounds formation in lipids and stimulated PARP activity, reflecting increased free radical damage of DNA. Amyloid beta and H_20_2 significantly enhance intrasynaptosomal protein oxidation but had no effect on extrasynaptosomal membrane protein oxidation. Several antioxidants decrease protein oxidation determined by dityrosine formation in synaptosomal proteins, however, the results are not statistically significant.

Our results indicated, that the early oxidative damage induced by Aβ 25-35 is DNA damage. Full length Aβ 1-40 stimulates significantly not only DNA damage and in consequence PARP activity, but also membrane lipid and protein oxidation that may be responsible for nerve endings degeneration and cell death.

Aβ activates proapoptotic factor(s) in brain synaptosomes

Contributors: Agata Zambrzycka, Grzegorz Czapski, Joanna B. Strosznajder

In the following reported study the effect of Aβ on the induction of apoptotic factor(s) in cytosolic fraction of synaptosomes was investigated. The synaptosomal fraction was incubated for 4 h with Aβ 25-35 at 25μM. Subsequently synaptosomal cytosol was obtained and was incubated with nuclear fraction for 30-120 min in the absence and presence of different inhibitor(s) chelator of Ca^{2+} and antioxidants. DNA integrity was evaluated by agarose electrophoresis. The preliminary data indicated that Ab 25-35 enhances significantly the influx of Ca^{2+} into synaptoneurosomes through L-type voltage operated calcium channels. Moreover, Ca^{2+}- dependent cytosolic apoptotic pathway was activated by Ab 25-35 peptides in synaptosomes. Inhibitor of caspase-3 (Ac-DEVD-CMK) at 100μM decreased amyloid-beta evoked DNA fragmentation. Our results have shown that Aβ peptide through stimulation of Ca^{2+} influx and Ca^{2+} dependent processes in synaptosomal cytosol activates DNA degradation. Aβ itself even in the presence of high Ca^{2+} concentration not disturbed DNA integrity.

Molecular processes of cells degeneration during oxidative stress *in vitro* and after brain ischemia

Contributors: Grzegorz Czapski, Małgorzata Chalimoniuk, Joanna B. Strosznajder

In order to better understand the effects of oxidative stress that occurs during reperfusion after brain ischemia, the protein oxidations in endothelial cells in culture and in nerve endings fractions were evaluated. In this study the new fluorescence probes Tyr-fluo were introduced for the determination of thyrosine oxidation and dithyrosine formation in extracellular and intracellular compartments. The study on the effect of activated neutrophils on protein oxidation of

endothelial cells in culture and on the role of nitric oxide donors was completed in 2001. The oxidative stress *in vitro* enhances protein oxidation and it is suggested that oxidation of phosphatidylinositol transport protein (PI-TP) may be responsible for the alteration of these proteins during reperfusion after brain ischemia. The study on PI-TP has been completed and the paper is being published.

During brain ischemia and inflammation, purine nucleotides are released together with the neurotransmitters and exert their effect through specific purinergic receptors. The molecular mechanism of effect of ATP on receptor mediated NO release was investigated. It was observed that activation of purinergic receptors by specific agonist had no direct effect on NO synthesis and that the purinergic nucleotides enhanced INFα-evoked induction of inducible isoform of NOS. The part of this subject is realised as the grant NIH project.

THE ROLE OF Aβ PEPTIDE IN MODULATION OF ChAT ACTIVITY, PHOSPHOINOSITIDES KINASE AND PHOSPHOLIPASE C IN ADULT AND AGED BRAIN

Supported by the State Committee for Scientific Research: grant # 4 P05A 072 19

Project leader: Joanna B. Strosznajder
Contributor: Agata Zambrzycka

Choline acetyltransferase (ChAT, acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6), involved in the learning and memory processes is responsible for the synthesis of acetylcholine. There are many discrepancies in literature concerning ChAT activity during brain aging and the role of amyloid beta peptides in modulation of this enzyme. The study on the mechanism of ChAT regulation and age-related alterations of ChAT activity in different parts of the brain was continued. Moreover the molecular mechanism of Ab peptides on ChAT activity in adult and aged brain was studied. The enzyme activity was determined in the brain cortex, hippocampus and striatum in adult (4-months-old) and old (24-months-old) animals. The highest activity was observed in the striatum. We found that the enzyme

is not regulated by phosphorylation-dephosphorylation processes and is not dependent on calcium ions, but is sensitive to oxidative stress in the striatum. About 70% of the total ChAT activity is present in the cytosol. Arachidonic acid significantly inhibited cytosolic form of this enzyme. In the brain cortex and striatum from aged brain, ChAT activity was inhibited by 50% and 37%, respectively. The aggregated form of Aβ 25-35 inhibited ChAT activity only in the aged striatum without effect on the enzyme in adult brain. The results from our study indicate that aging processes play a major role in the inhibition of ChAT activity. The mechanism of inhibition seems to be related to enhanced level of free arachidonic acid and reactive oxygen species. In further studies, the effect of Aβ 25-35 on the phosphoinositides kinases and on the expression of different isoforms of phospholipase C (PLC) β, γ, δ and on PLC activity was investigated. It was observed that aggregated form of Aβ peptides decreases selectively the phosphatidylinositol kinase (PI-kinase), the key enzyme in the synthesis of polyphosphoinositides (PIP and PIP2).

MOLECULAR MECHANISM OF iNOS INDUCTION IN BRAIN CELLS

Supported by the National Institute of Health, USA: Fogarthy International Research Collaboration Award (FIRCA) # PA-95-011"Chronic alcohol on cholinergic signaling pathway and NOS"

Project leaders: Grace Y. Sun*, Joanna B. Strosznajder
Contributors: Małgorzata Chalimoniuk, Robert P. Strosznajder

In the brain microglia, cells play an important role in mediating inflammatory responses in ischemic brain and in a number of neurodegenerative diseases. A major mechanism is their ability to respond to cytokines that evoke expression of inducible nitric oxide synthase (iNOS). Microglia express purinergic receptors, namely, $P2X_{1,4,7}$ and $P2Y_{1,2,6}$ receptor subtypes, which can be implicated in the regulation of purine nucleotides release during neuronal injury. In this study,

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the effect of extracellular nucleotides such as ATP and its analogues, BzATP, MeSATP and ADP on NO release in the BV-2 microglia cells in culture was investigated. These nucleotides itself did not induce iNOS expression. However, all of them enhanced INF-γ induced iNOS expression and NO production. Oxidized adenosine 5-trisphosphate (o-ATP), the P2X7 receptor inhibitor, decreased in a dose dependent manner the ATP and BzATP mediated NO production. The effect of ATP on IFNγ-induced NO production was also inhibited by a non-specific P2 receptor antagonist suramin, but not by the antagonist of $P2Y_1$ receptor. The further study indicated that activation of PKC- and ERK1/2-dependent NFκB pathway and the JAK/STAT pathway is essential for IFNγ-induced iNOS in BV-2 microglia cells. Inhibitors of these kinases were effective in suppression of both IFNγ and IFNγ-ATP induced NO production. Western blot analysis showed that BzATP was more effective in enhancing IFNγ-induced ERK1/2 phosphorylation than other nucleotides. These data suggest that extracellular purine nucleotides enhance IFNγ-induced NO production in BV-2 microglia cells through stimulation of the P2X7 receptor.

EFFECT OF DOPAMINERGIC RECEPTORS D1/D2 ACTIVATION ON GLUTAMATERGIC DEPENDENT EXPRESSION OF NO SYNTHASES AND G-KINASE IN PATIENTS WITH IDIOPATHIC PARKINSON DISEASE (PD)

Supported by Grant of Institute of Aviation Medicine, Warsaw # 62/00/S

Project leader: Adam Stępień*
Contributors: Małgorzata Chalimoniuk, Joanna B. Strosznajder

The aim of this study was to investigate the serum prooxidants and antioxidants level and the activity of antioxidant enzymes in the blood of patients with PD treated with levodopa and with levodopa plus pergolide mesilate – D2 dopaminergic receptor agonist. The results of this study suggest that the increased free radical levels and lipid peroxidation in this disease could be due to oxidative stress

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and that pergolide mesilate, D2 receptor agonist, decreased oxidative stress through enhancement of antioxidant enzymes activity such as SOD and catalase. Agonist of D2 receptor may exert its protective effect not only by stimulation of catalase and SOD activity but also by enhancement of GSH level. These results suggest that D2 receptor agonist is a more efficient in decreasing of oxidative damage in PD comparing to levodopa.

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CHANGES OF TRANSPORT OF NEUROACTIVE AMINO ACIDS IN HYPERAMMONEMIC ENCEPHALOPATHY: ROLES IN NEUROTOXICITY AND NEUROPROTECTION

Project leader: Jan Albrecht
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Recent *in vitro* evidence suggests that the nonproteinaceous sulfur amino acid taurine (Tau) added at ~ 1-20 mM concentration to brain slices or cultured neurons prevents cell damage incurred by excitotoxin overactivating the ionotropic (NMDA or KA) glutamate receptors. However, the antiexcitotoxic potential of Tau has not been demonstrated *in vivo* as yet. Ammonia administered i.p. into rats excessively activates NMDA receptors, and the severity of the ensuing neurologic symptoms is correlated with the activity of NO synthesis as indexed by extracellular accumulation of cGMP in brain microdialysates. We set to determine whether ammonia infused to striatum *via* microdialysis tube will promote cGMP accumulation in the striatal microdialysates and whether Tau added by the same route would prevent this effect.

The study has shown, that: a) in the absence of ammonia, cGMP level in the microdialysates remained at a very low level throughout the whole infusion period, both in the presence and absence of Tau; b) ammonia alone produced a dramatic increase of the cGMP content in the microdialysates; c) the effect of ammonia was completely abolished by coadministration of Tau. The results constitute the first evidence of an excitotoxic effect of exogenous Tau *in vivo*. Tau as such cannot be used to treat or prevent the excitotoxic effects asso-

ciated with a variety of pathologic conditions, including hyperammonemia, because it very poorly crosses the blood-brain barrier and this is not altered by hyperammonemia. However, the application of Tau derivatives that do penetrate the blood-brain barrier remains a possibility worth testing.

Accumulation of taurine (Tau), glutamate (Glu) and glutamine (Gln) was measured in vivo in microdialysates of the striatum following a direct application to the microdialysis tube of 60 mM ammonium chloride which renders the final ammonia concentration in the extracellular space to ~ 5mM. The following compounds were coadministered with ammonia to distinguish between the different mechanism that may underlie the accumulation of amino acids: ion transport inhibitors, diisothiocyanostilbene-2,2'-disulfonate (DIDS) and furosemide, a Glu transport inhibitor L-transpyrrolidine-2,4-dicarboxylate (PDC), an NMDA receptor antagonist dizocilpine (MK-801) and an 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate (KA) receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX). Ammonia stimulated Tau accumulation in the microdialysates to ~ 250% of the basal value. Furosemide did not significantly affect the stimulation by ammonia and DIDS only moderately depressed the effect. The ammonia-dependent Tau accumulation was increased by ~ 50% in the presence of PDC and reduced by ~ 35% in the presence of dizocilpine and DNQX. In the microdialysates ammonia stimulated Glu and Gln accumulation somewhat less than Tau accumulation. Except for stimulation of Gln accumulation by DNQX, the effects were not modified by any of the cotreatments. The results are consistent with the assumption that ammonia stimulates Tau efflux mainly *via* activation of ionotropic Glu receptors.

As mentioned in the preceding paragraph, the acute effects of ammonia are excitotoxic, and are mediated by excessive activation of the NMDA class of glutamate (Glu) receptors, subsequent free radical formation and enhanced NO synthesis. On the other hand, studies *in vitro* and *in vivo* have indicated that the cytotoxic component of ammonia-induced cerebral edema is associated mainly with astrocytic swelling. In the present study we investigated the possible

association between the excitotoxic effects of ammonia and the accompanying cell swelling. We also assessed the relative role of Gln accumulation, which is held responsible for the osmotic mechanism of the ammonia induced cerebral edema.

Incubation with 5 mM ammonium acetate ("ammonia") increased ^{14}C inulin space reflecting cell volume, and Gln content in rat cerebral cortical slices. An NMDA receptor antagonist - MK-801, a nitric oxide synthase inhibitor - NNA, and an antioxidant, taurine (Tau) markedly attenuated the cell volume-increasing effect of ammonia, but did not reduce Gln content. MK-801 was relatively ineffective in reducing ammonia-induced cell swelling at acidic pH, consistent with its reduced ability to interact with the NMDA receptor channel under these conditions. The effect of 10 mM Tau was abolished by GABA(A) receptor antagonist, biccuculine, but was not affected by a Tau transport inhibitor GES, and was not mimicked by a neuroinert osmolyte, 10 mM mannitol, pointing to the cell membrane as a locus of the antiexcitotoxic action of this amino acid. The results indicate that swelling of CNS cells by ammonia, a major cause of brain edema in hyperammonemic encephalopathies, is mechanistically related to ammonia-induced excitotoxicity and partly independent of the osmotic effects of Gln.

Collaborating units

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REGULATION OF KYNURENIC ACID SYNTHESIS IN C6 GLIOMA CELLS IN CULTURE

Project leader: Jan Albrecht
Contributors: Jan Albrecht, Wojciech Hilgier, Monika Dolińska

Studies with brain slices have provided evidence that synthesis of kynurenic acid (KYNA) from kynurenine (KYN), which occurs in astrocytes, is modulated by changes in the ionic composition of the medium, and the presence of depolarizing agents or the excitatory amino acid glutamate (Glu). The present study analyzed the effects of changes in incubation medium on KYNA synthesis in cultured C6 glioma cells. The synthesis was not affected by omission of Na^+ and raising K^+ concentration to 50 mM – conditions which in brain slices stimulate or inhibit KYNA formation, respectively. KYNA synthesis in C6 cells was inhibited by the absence of Ca^{2+}, which contrasts with its Ca^{2+}-independence in brain slices. Lack of Mg^{2+}, and addition of a chloride channel blocker, 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonate (SITS) did not affect the synthesis either. KYNA synthesis in C6 cells was done dependently inhibited by Glu. The inhibitory effect of Glu was not prevailing in C6 cells, suggesting that Glu acted intracellularly. NH_4Cl and veratridine decreased affected by GDPßS, an antagonist of metabotropic Glu receptors, the receptor class KYNA production, mirroring the effects noted with brain slices. KYNA synthesis was strongly reduced in the presence of leucine (Leu), and the uptake of [^{14}C]Leu was inhibited by the KYNA precursor KYN, which points to Leu as potential endogenous modulator of KYNA formation in CNS cells.

Collaborating units

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MECHANISM AND REGULATION OF CELL MEMBRANE GLUTAMINE TRANSPORT

Supported by the State Committee for Scientific Research; grant # 4 PO5A 060 18

Project leader: Jan Albrecht
Contributors: Jan Albrecht, Monika Dolińska, Anna Dybel

The uptake of [^{3}H]Gln in mouse cortical astrocytes and cerebellar neurons was examined with regards to its compatibility with the system transporting Gln in different tissues and with the expression of genes coding transporters specific or nonspecific for CNS. The major findings were as follows: a) an active system A-mediated transport was confined to neurons, and so was the expression of the GlnT transporter detected in neurons in rat brain sections. Accordingly, MeAiB a model system A substrate was taken up exclusively in astrocytes; b) the activity of system ASC-mediated transport was present in both cell types and was correlated with the expression of mouse, but not rat ASCT2 transporter; c) the system N-mediated transport was likewise present in astrocytes and neurons, as was the expression of NAT2 – a mouse retinal variant of the N system; d) SAT2 – an ubiquitous rat system A variant, was more strongly expressed in astrocytes than in neurons. The results show a significant, albeit incomplete cell type specific coordination of Gln transport activity with the expression of CNS-specific Gln transporters.

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Publications

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PATHOGENETIC MECHANISMS OF POSTISCHEMIC CHANGES IN GLOBAL AND FOCAL ISCHEMIA OF CENTRAL NERVOUS SYSTEM IN DIFFERENT EXPERIMENTAL MODELS AND IN HUMANS

Project leader: Janina Rafałowska
Contributors: Dorota Dziewulska, Roman Gadamski, Sławomir Januszewski, Ewa Matyja, Mirosław J. Mossakowski, Ewa Nagańska, Piotr Piotrowski, Ryszard Pluta, Grażyna Szumańska, Mieczysław Śmiałek, Anna Taraszewska, Krystyna Wierzbicka, Renata Wojda, Irmina B. Zelman.

Blood-brain barrier disturbances and morphological changes in rat brain after photochemically induced focal ischemia

Project leader: Roman Gadamski
Contributors: Grażyna Szumańska, Renata Wojda.

The aim of this study was the evaluation of our own model of focal cerebral damage caused by a photodynamic reaction and determination of its utility as a model of brain necrosis and blood-brain barrier damage in the rat. Wistar rats were used for the experiments. The animals were anesthetised with 3% chloral hydrate (325 mg/kg) and injected intravenously with 40 mg/kg of 3% solution of rose bengal. After removal of the periostium the brain was irradiated through the skull for 30 min with a 250 W halogen, air-cooled light source. The material for morphologic studies was sampled 24h, 4 days and 7 days after irradiation. Morphological and immunocytochemical examinations were performed. The results document the usefulness of this method for studying focal brain ischemia in rats. The observed

morphological changes and disturbances in blood-brain barrier provided information about the dynamics of the formation of gliosis and necrotic foci, and the quality and extent of brain damage in the surrounding tissue.

Neuronal death in the rat hippocampus in experimental diabetes and cerebral ischemia treated with antioxidants

Project leader: Piotr Piotrowski
Contributors: Mieczysław Śmiałek, Krystyna Wierzbicka

Male Wistar rats were subjected to intraperitoneal (i.p.) streptozotin (85 mg/kg) to evoke diabetes. Cerebral ischemia was produced by injection of 0.003 ml of air into the left carotid followed by bilateral common carotid ligation. We studied the effect of application of two antioxidants - coenzyme Q10 (CoQ10, 10 mg/kg b.w., i.p. for seven days) and lipoic acid (LA, 100 mg/kg b.w.,i.p. for seven days) on neurons and on the apoptosis-related enzyme - caspase-3 activity in the hippocampus and dentate gyrus. Ischemia and diabetes led to a decrease of nuclear and perikaryon diameters as well as neuronal density in the CA1, CA2, CA3 and dentate gyrus. Application of CoQ10 or LA for seven days improved the mean nucleus area and perikaryon area in almost all investigated structures. Both antioxidants diminished neuronal loss in the diabetes complicated with ischemia but not in the animals with diabetes only. Activity of one of the key enzymes in apoptotic cell death, caspase-3 (CPP32), increased in hippocampus in the diabetic rats, in the animals with cerebral ischemia and in the rats with both diabetes and ischemia by about 80%, 33% and 53%, respectively. Either the CoQ10 or the LA treatment led to a significant decrease of CPP32 activity in all experimental groups. The effects of the antioxidative treatment support the hypothesis of the important role of oxidative stress and free radicals in neuronal pathology in diabetes and ischemia. The above results of CPP32 activity suggest the important role of apoptosis as a mechanism of cell death and demonstrate the positive effect of the CoQ10 or LA treatment.

Changes in the distribution of sugar receptors in the brain of Mongolian hamster induced by 5-minute ischemia

Project leader: Grażyna Szumańska
Contributor: Jolanta Krzywicka

The aim of the present study was to investigate changes in the localisation of glycoconjugates caused by 5-minute ischemia as a consequence of bilateral ligation of common carotid arteries. Histochemical evaluation was performed using specific lectins that recognize the following sugar residues: 1) α-D-galactosyl and N-acetylgalactosaminyl (BS-I), 2) N-acetyl-β-D-galactosaminyl (RCA-60), 3) N-acetyl-α-D-galactosaminyl (HPA), 4) α-D-mannosyl and α-D-glucosyl (Con A), 5) α-D-fucosyl (TG). It was found that the first two lectins (BS-I and RCA-60) bound selectively to the endothelium, while two others (HPA and ConA) showed affinity predominantly to cellular compartments of the brain, like neurons and glia, but also to neuropil and the white matter. TG agglutinin did not stain any brain structures. Significant changes in the arrangement of examined glycoconjugates as a result of ischemia were observed. First, the intensity of binding of BS-1 and RCA-60 to their complementary residues was increased in the capillary network as compared with control animals. Glycoconjugates recognised by these lectins were also found in reactive astrocytes in various brain structures. Second, the number of sugar receptors visualised by Con A and HPA was enhanced in the majority of cellular compartments of the brain and, though to a lesser extent, in terminal capillary network, especially in the neocortex, while in control animals these sugar receptors were detected in brain microvessels. Redistribution of particular sugar residues after ischemia suggests that functional changes associated with this type of injury might take place in various CNS structures.

Phenotypic characteristics of GFAP positive oligodendroglial tumors

Project leader: Ewa Matyja
Contributors: Anna Taraszewska, Ewa Nagańska

Oligodendrogliomas are believed to derive from oligodendrocyte lineage but the expression of different immunohistochemical markers indicates some variability in their differentiation potency. In particular, the evidence of an astrocytic differentiation in oligodendrogliomas has been a subject of considerable debate. An immunohistochemical and ultrastructural study was performed to evaluate the phenotypic characteristics of anaplastic oligodendrogliomas and mixed oligoastrocytomas containing GFAP-immunopositive tumor cells. GFAP-positivity was shown in a number of morphologically different neoplastic cells, responding to typical neoplastic oligodendrocytes (gliofibrillary oligodendrocytes-GFOC), miniature form of gemistocytes (minigemistocytes) and neoplastic or reactive astrocytes. The electron microscopic study of anaplastic GFAP-positive oligodendrogliomas revealed cell variability particularly with reference to astrocytic differentiation. The majority of neoplastic cells have shown the fine structural features of oligodendrocytes, accompanied by various amounts of intermediate cytoplasmic filaments corresponding closely to the perinuclear rim of GFAP-immunoreactivity. These cells exhibited features common for both oligodendroglial and astroglial cells and might be regarded as an intermediate morphological form between these two cell lineage. The data of our immunohistochemical and ultrastructural findings support the opinion that oligodendroglial tumors seem to be derived from the progenitor cells which are able to differentiate into either oligodendroglial or astroglial cells. GFAP-immunoreactive intermediate filaments in neoplastic oligodendroglial cells seem to be widely distributed and might have important practical implications for diagnostic neuropathology.

A case of cystic form of angiomatous meningioma with prominent microvascular pattern mimicking hemangioblastoma

Project co-ordinator: Anna Taraszewska

A surgical case was reported of a rare cystic form of angiomatous meningioma with a predominant microvascular pattern mimicking hemangioblastoma. This case was used to exemplify some of the difficulties in histopathological classifications of highly vascular tumors of the meninges with variable histogenesis and malignancy. Alterations over years in terminology of these tumors have been stressed.

Ultrastructural characteristics of necrotic and apoptotic mode of neuronal cell death in a model of anoxia *in vitro*

Project leader: Ewa Nagańska
Contributor: Ewa Matyja

The morphological pattern of postanoxic changes has been widely studied in various experimental models, however, the exact mechanism of neuronal cell death induced by ischemic/anoxic insult is still not fully understood. Thus, the study was performed to determine the detailed ultrastructural criteria of postanoxic neuronal changes in model of anoxia *in vitro*. The electron-microscopic examination of organotypic cultures of rat hippocampus exposed to 10 and 20 minutes of anoxia revealed the morphological features typical for both necrotic and apoptotic neuronal cell death. Numerous neurons revealed a typical picture of passive necrotic lysis such as advanced swelling of intracellular organelles associated with cell membranes disruption. Others clearly reflected an active apoptotic form of cell injury consisting of condensation of nuclear chromatin with early preservation of cell membranes. However, there was also a subset of damaged cells sharing several features typical for both necrosis and

apoptosis. These results add additional evidence to the previous studies suggesting that neurons injured by anoxic insult can die not only in a pure necrotic or apoptotic way but a continuum between apoptosis and necrosis in certain pathological conditions might exist.

Encephalopathy and other vascular neurological syndromes with familial occurrence

Project co-ordinator: Janina Rafałowska

A review is presented of literature data concerning vascular diseases occurring familialy. They manifest clinically as recurrent TIA, ischemic and hemorrhagic strokes and other blood supply disturbances and lead to numerous vasogenic brain tisssue damage of varied intensity. Particularly evident lesions are observed in the hemispheric white matter. Progressive neurological symptoms and dementia form the picture of subcortical leucoencephalopathy in several family members. Moyamoya disease, fibromuscular dysplasia, hereditary hemorrhagic teleangiectasia, hereditary cerebral hemorrhage with amyloidosis, *pseudoxanthoma elasticum*, CADASIL, two types of subcortical encephalopathy in Japan, and HERNS are described.

Pathogenic factors and tissue reactivity in aging

Project co-ordinator: Janina Rafałowska

The process of aging affects organelles of cells, their membranes, metabolic resources, synapses, receptors, changes in activity or synthesis of enzymes, cytokines, trophic factors and neurotrasmitters. Although the activity (or number) of some of these factors increases, but the metabolism decreases. Different metabolism of old tissues causes different responses to pathogenic agents, and often another course of a disease. Different metabolism also causes other reaction to drugs. In old men, a decrease in drug dosage is often effective. However, metrical age does not always correspond with

biological age. The process of both development and aging runs differently in various individuals.

Early ontogenic disturbances in cell migration in mentally disabled adult – case report

Project leader: Janina Rafałowska
Contributor: Dorota Dziewulska

Disturbances in cell migration are heterogenic disorders of brain development, commonly associated with epilepsy and mental retardation. A 45-year-old oligophrenic man with a defect of lower limbs and a family history of mental retardation who died because of brainstem hemorrhage was observed. At the post-mortem and histopatological examination, a complex brain malformation characterized by bilateral periventricular heterotopia, cortical dysgenesia, partial agenesia of corpus callosum, and thin-walled blood vessels was found. The immunohistochemical examination revealed a presence of fibronectin, collagen IV and laminin in walls of pathological vessels. Cyclooxygenase-2 (COX-2) expression in neurons of the heterotopias and dysgenic cortex was negative. The lack of the COX-2 expression may indicate the maturity of these neurons and indirectly, the normal activity of postsynaptic NMDA receptors, which explains the absence of epileptic attacks in our patient. The COX-2-negative neurons and compounds of basal lamina in fetal-like vessels also suggest that these neural structures matured during the 45 years of the patient`s life.

Are all new data referring to amyotrophic lateral sclerosis certain? Some doubts

Project co-ordinator: Janina Rafałowska

Progress in molecular investigations produces new data that facilitate the recognition of pathogenesis mechanisms of numerous nervous system diseases, among them amyotrophic lateral sclero-

sis (ALS). Molecular studies of ALS mainly concentrate on genetic search, excitotoxicity and astrocytic participation, selective motoneuron degeneration. In literature, part of the results of these investigations are presented as definite, but some doubts exist. These doubts concern the role of the superoxide dismutase (SOD1) mutation gene and free radical scavenging, because it is not clear how the SOD1 mutation gene acts. It is known, that in ALS not only motoneurons, but also cells of the sensory system die. Morphological changes in ALS are considerably wider than a manifestation of clinical symptomatology. It seems that the "selective death" of motoneurons limits the problem of ALS pathogenesis. It seems also that free radicals in the SOD1 mutation gene as well as excitotoxicity (and other factors) are not etiological in ALS. They play a great role only in the disease pathogenesis as metabolic cascade factors, which in various disease processes are observed.

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Publications

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STUDIES ON THE PATHOMECHANISM AND EFFECTS OF BRAIN AND HEART ISCHEMIA WITH PARTICULAR REGARD TO DISTURBANCES OF CEREBRAL BLOOD FLOW REGULATION

Pathomechanisms of heart and brain ischemia

Project leader: Andrzej Kapuściński
Contributors: Ewa Koźniewska-Kołodziejska, Robert P. Ostrowski, Sławomir Januszewski, Zdzisława Kowalska, Lidia Radomska

The experimental cardiology was the new scope of our research introduced in 2001. The goal of the study was to evaluate the effect of endothelin receptor antagonist on plasma leptin level after myocardial infarct (MI). Leptin has been shown to be a strong predictor of heart disease. The experimental model of MI was elaborated and implemented after left thoracotomy by ligation of the distal part of the left coronary artery (CA). The experiments have been performed within the Principles of Laboratory Animal Care formulated by the Local Ethic Council for Medical Research (agreement No 97, 8.07.2001). The particular objectives included analysis of ECG records, quantitative evaluation of infarct size by means of computer techniques (morphometry using image analyzer CUE-3, Olympus and/or scanning with quantitative analysis applying software Photoshop 6.0 CE), assessment of leptin concentration in plasma (by means of radioommunoassay, RIA with I-125 labelled antigen), and effect of endothelin receptor antagonist (Tracleer TM - bosentan, Actelion Pharmaceuticals Ltd) on plasma leptin concentration. Experiments were performed under chloral hydrate anesthesia in

several experimental groups: control, MI not treated, MI treated with bosentan given daily by gavage. Results confirmed development of MI after occlusion of CA, revealed significant effect of bosentan on decrease of leptin concentration in plasma and diminution of mortality in bosentan treated group during 48 h after MI. The results might indicate that bosentan has an effect on leptin concentration in cardiovascular pathology.

In collaboration with Laboratory of Pathobiochemistry of the CNS, changes in the GABA transport in synaptosomes and in kinetics of $GABA_B$ receptor were evaluated in cerebral ischemia after cardiac arrest. The effect of prolonged perfusion on advancement of alterations in hippocampus after periodical global brain ischemia was evaluated by means of morphometric methods.

Impact of brain ischemia on the regulation of cerebral blood flow

Project co-ordinator: Ewa Koźniewska-Kołodziejska

Studies on the regulation of cerebral microcirculation after focal cerebral ischemia/reperfusion concentrated on the participation of nitric oxide (NO) in hyperperfusion observed upon reperfusion of the brain after 30 minutes of middle cerebral artery occlusion (MCAO) in rats. Pretreatment of the animals prior to MCAO with either nonspecific inhibitor of NO synthase or specific neuronal synthase inhibitor, did not affect the severity of ischemia as assessed with laser Doppler flowmetry but modulated the time course and magnitude of hyperperfusion. Comparison of the effects of both inhibitors suggests that endothelial isoform of NO has more important contribution to hyperperfusion than the neuronal one.

This year we have started an informal cooperation with the Department of Clinical Neurosciences, Brown University School of Medicine, Providence, RI, USA. The aim of the common study concerns participation of vascular endothelial growth factor (VEGF) in CNS injury after permanent ischemia, mechanical trauma and subarach-

noid hemorrhage. VEGF is known not only as a growth factor essential for angiogenesis but also as a permeability factor promoting vasogenic brain edema. Our hitherto results suggest that following brain trauma (TBI) VEGF is expressed in various cell populations within CNS at different times after TBI. VEGF positive cells appear on the injured site as early as a few hours after the insult. This early appearance of VEGF correlates with the accumulation of water in the brain. The second strong VEGF signal which peaks with a delay of 4-days and represents the expression of VEGF mainly in astrocytes is most probably connected with the formation of new blood vessels.

Collaborating units

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Publications

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MILD COGNITIVE IMPAIRMENT (MCI)-CONVERSION INTO THE PRIMARY DEGENERATIVE DISEASE AND VASCULAR DEMENTIA. GENETIC, BIOCHEMICAL AND CLINICAL STUDY

Project leader: Maria Barcikowska
Contributors: Tomasz Gabryelewicz, Marek Gołębiowski, Beata Pepłońska, Anna Pfeffer-Baczuk, Jarosław Pniewski, Dorota Religa, Maria Styczyńska, Bogusław Wasiak

In 2001 we organized a DNA bank for neurodegenerative diseases. Polymorphism of different genes was studied according to their influence on clinical picture and cognitive and non cognitive disturbances in Alzheimer's disease (AD). Apolipoprotein E (APO E) genotype and behavioral pathology have not been involved according to our observations. We have demonstrated a lack of relationship between the presence of the APO Eε4 allele and behavioral symptoms, including delusions, hallucinations, depression, activity disturbances, aggressiveness and anxiety. There was no statistically significant correlation between APO E genotype and the results of *in vivo* measurements of temporal lobe atrophy, which was probably due to the completely different nature of both tests. We have also had the possibility to observe two patients with early onset AD and unique APO E ε 2/2 genotype. In contrary, the results of neuropsychological assessment and polymorphism of APO E have shown that several cognitive processes depend on the APO E genotype . Patients with ε4 allele had greater deficit in delayed recall of new information. On the other hand, working memory appeared to be more impaired in the non ε4-group patients. We examined not only genetic pathology but also the vascular changes as an important

factor in the development of dementia and as an enhancer of amyloidogenic changes in AD. We studied the relationship between total homocysteine (tHcy) levels in AD patients and vascular pathology in brain imaging. In the group of patients with radiological changes, plasma tHcy level was significantly higher (18.79 +/- 8.28), than in patients without evidence of vascular lesions (16. 24 +/- 10.40), p=0.008. We concluded that plasma tHcy level may have been an independent vascular risk factor in our group of AD patients.

ONGOING SIMULTANEOUS ANALYSIS OF APOE, α2MACROGLOBULIN AND CATHEPSIN D AS MARKERS OF GENETIC POLYMORPHISM IN A SELECTED GROUP OF POLISH AD PATIENTS

Supported by the State Committee for Scientific Research: grant # 4 P05B 133 19

Project leader: Maria Barcikowska
Contributor: Maria Styczyńska

In the cohort of 100 patients (F:66, M:34; mean age 76.4+/- 4.7; mean age of onset 71.0 +/- 4.6) recruited from the Department of Neurodegenerative Disorders in Warsaw we manage to confirm the association only between APOε4 allele and the risk of AD. We didn't find statistically significant increase in frequency of α2M-1000 Val allele and cat*D T allele in AD group, even though this effect was reported by some authors.

MILD COGNITION IMPAIRMENT AS A DEMENTIA RISK FACTOR

Supported by the State Committee for Scientific Research: grant # 6 P05B 058 20

Project leader: Tomasz Gabryelewicz
Contributors: Maria Barcikowska, Anna Pfeffer, Maria Styczyńska, Dorota Religa, Elżbieta Łuczywek, Bogusław Wasiak

The aim of the study is to evaluate the risk factors for developing dementia among the 100 persons with Mild Cognitive Impairment

(MCI) within three years of observation. Project was started in May 2001. We diagnosed 70 persons. Every patient was assessed by a neurologist, a neuropsychologist and a psychiatrist. CT, SPECT and genetic examination of known risk factors were performed.

Publications

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ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL STUDIES OF THE BRAIN DEVELOPMENTAL DISORDERS. STUDY OF INFLAMMATORY PROCESS

Project leader: Danuta Maślińska
Contributors: Milena Laure-Kamionowska, Agnieszka Kaliszek

Cells, participating in the inflammatory process produce cytokines, that constitute an inflammatory "network" amongst such cells. Responsible for modulating the duration and intensity of inflammation, cytokines come under close scrutiny as a possible target of therapeutic intervention. In the brain, the presence of proinflammatory cytokines is observed regardless of where the inflammation process actually takes place: within or beyond the central nervous system. One of the cytokines playing a vital role in the modulation of inflammation is interleukin 17 (IL-17). Although it is synthetised by a subpopulation of Th1 lymphocytes, IL-17 acting on the specific receptors localized on different types of cells is able to regulate the synthesis of a number of cytokines. Our study was performed on two experimental models: a/ animals in which an inflammatory process was induced through the intravenous administration of *Staphylococcus aureus* α-toxin; b/ animals in which the inflammation was induced in the brain itself, *via* middle cerebral artery occlusion. The results showed, that immediately after each of the procedures was carried out, the number of blood cells displaying IL-17 expression increased significantly, but in the brains it appeared after a few days' delay. In animals intoxicated with α-toxin, IL-17 was found in the perivascular fluid (immunohistochemistry) and it was associated with

the ultrastructural changes of the brain vessel wall. In the brains of animals affected by ischemia the expression of the IL-17 protein was detected within numerous blood derived cells infiltrating an injured brain parenchyma. Our results documented, that IL-17 synthesis in peripheral blood precedes the appearance of this interleukin in the brains and suggest that the therapeutic inhibition of it within a few days span of the damaging factor action, may successfully limit proinflammatory influence of IL-17 on the brain parenchyma.

We continued the study on the constitutive expression of two IL-15 mRNA isoforms in human developing brains. The results showed, that the expression of both interleukin isoforms changes in various brain structures in the course of development. Our results documented that in those structures the IL-15 receptor α subunits mRNA are present. Obtained data supports our previous observations that IL-15 is involved in the growth and differentiation of the human brain.

Perinatal asphyxia is the most common cause of brain injuries that leads to death of newborns and disabilities in surviving children. Characteristic for these injuries are cavities in which the healing process is impaired (porencephaly). Since recent observations proved that leptin may accelerate wound healing in some tissues, we have studied the presence of the specific leptin receptors on astrocytes participating in the brain healing process. The results showed that astrocytes localized in the injured brain hemisphere of newborn rats affected by asphyxia, express the protein of the specific, long leptin receptor OB-Rb, that was previously recognized as a functional one. It suggests that as in other tissues leptin may play a role in the healing process of the injured brain parenchyma.

DEVELOPMENT OF THE NERVOUS SYSTEM AND PATHOLOGICAL CHANGES DURING THIS PERIOD – COMPARISON OF PHYLOGENETICALLY DIFFERENT STRUCTURES

Project leader: Maria Dąmbska
Contributor: Milena Laure-Kamionowska

A comparison of lesions in the central nervous system induced by anoxic-ischemic or infection factors was performed. A group of 20 brains of newborns and small infants with classic neuropathological stainings and GFAP reactions was investigated. The results showed the topography of lesions dependent on the developmental age of individuals, particularly in the periventricular area and early perivascular infiltrations in infected cases.

The ultrastructural study of experimental hypoxia (as a part of the Levine model of lesions) in immature rat brains which was partly performed in the previous year was finally accomplished demonstrating evident lesions in the cerebellum of experimental animals with accumulation of calcium deposits in nerve and glial cells as a part of a pathologic syndrom.

A case of early occurring disturbances of migration and maturation of nerve cells allowed to analyse this very rare type of anomaly.

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Publications

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SUBCELLULAR LOCALIZATION OF PROTEINS DURING APOPTOSIS: EXPERIMENTS WITH NOVEL ELECTRON MICROSCOPY TECHNIQUES. ULTRASTRUCTURAL ANALYSIS OF THE CEREBRO-VASCULAR JUNCTION AFTER TRAUMA

Project leader: Barbara Gajkowska
Contributors: Michał Walski, Hanna Olszewska-Bądarczuk, Małgorzata Frontczak-Baniewicz, Urszula Wojewódzka

The aim of the investigations in 2001 was to analyse the subcellular distribution of selected apoptotic proteins in a cell line COLO-205. Methods using post-embedding immunogold labeling and embedment-free electron microscopy (EFEM) were employed. At this stage we focused mainly on the proteins from the bcl-2 family: Bax, Bid, Bad.

We detected a rapid redistribution of the proteins from the cytoplasm to the mitochondria and later to the cell nucleus. This is the first demonstration of the nuclear homing of bcl-2 proteins. Using a double immunocytochemical labeling techniques, a co-localization of Bax and Bid was detected in some areas of mitochondrial membranes and in the cytoplasm, AG membranes, nuclear pores and finally in the nucleus. It is conceivable that Bid (which consists of the BH3 death domain and is the most potent bcl-2-like proapoptotic protein) dimerizes with Bax *via* an activatory interaction and facilitates its translocation from the cytosol to the membranous organelles. The above observations were confirmed by a fluorescence-based technique, laser scanning cytometry (LSC) which quantitatively determines fluorescence intensity on a cell-by-cell basis.

Employing EFEM and immunogold labeling we were able to demonstrate a structural interaction of Bax and Bid with the intermediate filaments of the cytoskeleton and with the filaments of the nuclear matrix. Moreover, both proteins were present in the mitochondrial matrix.

We initiated studies on the subcellular localization of the cytochrome c-induced pro-apoptotic proteases (caspases). Immunocytochemistry showed that CPP-32 is induced in the cytoplasm, especially in the perinuclear area in the cells committed to apoptosis. By double labeling techniques we were unable to show any co-localization of CPP and Bax.

EFEM showed the presence of CPP-32 on the intermediate filaments, especially on the filaments surrounding the nucleus. It is tempting to speculate that CPP marks the future incision sites on the filaments.

We continued our line of investigation on the pathology of the cerebro-vascular junction. At the present stage, we were interested in the effects of the trauma on the morphology of this junction. We conducted a detailed electron-microscopic analysis of the cerebral cortex and the pituitary 7 days after induction of trauma. We observed an induction of angiogenesis both in the cortex and in the neurohypophysis. New proliferating vessels formed a new vascular meshwork. Moreover, we detected a morphologically heterogenous population of the endothelial cells that on the early stages of development, filled the lumen of sprouting vessels. Later on, these cells flattened but their intercellular junctions were longer than in mature vessels. Vascular basement membranes were delaminated and often destroyed. Thus, brain trauma may precipitate angiogenesis, not only at the site of trauma but also in the remote brain regions.

THE ROLE OF IONIZED CALCIUM AND CALCIUM CHANNELS IN APOPTOSIS IN NORMAL AND CANCER CELLS. EXPERIMENTS USING FLUORESCENT CALCIUM DETERMINATION IN LIVING CELLS AND ELECTRON MICROSCOPY

Supported by the State Committee for Scientific Research: grant # 4 P05A 045 15

Project leader: Barbara Gajkowska
Contributors: Hanna Olszewska-Bądarczuk, Robert Gniadecki*

The aim of this study was to determine the involvement of intracellular calcium signaling and apoptosis with a focus on the alterations in the nucleus and nuclear matrix. We developed a unique electron microscopic technique EFEM which enables observation of the three-dimensional structure of the matrix. We described the presence of novel ultrathin filaments in nuclear matrix and the rearrangement of the matrix in the course of cell differentiation and apoptosis. By combining EFEM and immunocytochemistry it was feasible to follow the redistribution of the proapoptotic protein Bax from the cytoplasm to the nucleus. This new observation suggests the role of Bax in the nucleus. Morphological observations revealed the role of cytoplasmic ionized calcium in the condensation of chromatin. Measurements of Ca^{2+} with fluorescent probes and application of thapsigargin (blocker of calcium pump in the reticulum) and calcium chelators showed a role of intracellular calcium stores in the regulation of cell cycle and apoptosis. Emptying of calcium pool resulted in the cell cycle block and apoptosis independently of the concentration of Ca^{2+}_i in the cell.

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Publications

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CONTINUATION OF STUDIES CONCERNING DISTURBANCES OF INTRACRANIAL HOMEOSTASIS

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In December 2001 the Department of Neurosurgery was transferred from the District Hospital of Traumatology at Barska St. to Bielański Hospital. Under the new conditions, in the second half of the year the team was able to resume its previously conducted activities.

The doctorate works that had been started were completed.

In the doctoral thesis "Expression of nuclear T3 and retinoid receptors in gliomas" it has been found that the expression of genes encoding nuclear receptors of thyroid hormones – especially that of TRß gene – is distinctly reduced in patients with brain gliomas. Another finding in this group of patients was the overexpression of TRß1 receptor protein.

A thesis concerning "Morphometrical evaluation of CT images in the differential diagnosis between normal pressure hydrocephalus (NPH) and cerebral atrophia" has been completed. Based on the multi-level method for the NPH diagnosis, 78 patients with NPH were studied. A new diagnostic method to evaluate CT images by appli-

cation of an index referring to different CSF compartments was introduced.

Another doctoral work entitled "Possibilities of intracranial reserve evaluation by application of physiological loadings in an experimental model" – is in the last phase of preparation. Experiments were carried out on cats by intracerebral application of silicone imitating an ICP increase, similar to that caused by intracerebral hematoma. A clinical applicability of a diagnostic method based on response to physiological loadings was found.

In 2001, a new method of arachnoid cyst treatment by the non-valve connection between cyst and subarachnoid space at the lumbar level was introduced in clinical practice. The above mentioned operation was performed on 7 patients in the reported period.

Thirty five experiments on rats with a model of induced SAH were performed. Disturbances of cerebrovascular reactivity to CO_2 following SAH were investigated. Differences between supratentorial and infratentorial circulation were found (cooperation with dr E. Koźniewska from the Department of Nuclear Medicine).

Concerning neuropsychological aspects, two original papers and two case reports have been prepared. Analysis of differences in blood flow velocity in medial cerebral arteries during cognitive activity in young women and men showed bilateral brain activity in women and a predominance of left hemisphere activity in men in relation to many speech functions. Both case reports showed that the predominance of psychiatric symptoms in the clinical picture may lead to a delay in the diagnosis of the organic CNS disease that requires specific neurosurgical treatment.

In electrophysiological studies in 2001, EEG brain mapping images were found to be helpful in the determination of the epileptogenic focus location and in the evaluation of dynamics of bioelectric activity of the epileptogenic focus.

Approximately 1000 cases were verified by use of teleconsulting system enabling CT image transmission – the only one existing in Poland.

IN VITRO MR SPECTROSCOPY OF HIGH RESOLUTION IN AN INTRACEREBRAL TUMOR TISSUE AND CEREBROSPINAL FLUID – APPLICATION IN CLINICAL DIAGNOSTICS

Supported by the State Committee for Scientific Research: grant # 4 P05B 054 14

Project co-ordinator: Zbigniew Czernicki

Collection of the MR spectra pictures of the intracranial tumor tissue samples was continued. A method of High Resolution Magic Angle Spinning (HR-MAS MRS) was additionally applied to study the lyophilised tissues of brain tumors. According to literature this method has not previously been used. Spectral pictures of high quality, but completely different from those of tissue extracts were obtained. The applicability of HR-MAS MRS in the diagnosis of histological category of tumor has been evaluated.

EVALUATION OF INTRACRANIAL VOLUME - PRESSURE RELATIONS BY MEANS OF COMPUTERIZED TOMOGRAPHY IMAGING

Supported by the State Committee for Scientific Research: grant # 7T11E04020

Project co-ordinator: Jerzy Jurkiewicz

An above project is planned to be realized within 3 years. The first stage covered the period from the agreement signature till the end of the year 2001. The diagnostic station was created, the device was adapted and software to allow communication between different device components was installed. The scientific aim of the project is to create tools for non-invasive evaluation of volume – pressure relations in different states of intracranial pathology. We decided to correlate results of infusion tests with or without ICP measurement, mathematical analysis of CT images and cerebral blood flow measured by TCD, to achieve this goal. Measurement of parameters coming from different diagnostic devices required an efficient system of data acquisition designed for an off-line analysis.

The diagnostic station was adapted to the actual equipment of the Department. An additional mode for invasive blood pressure measurement was incorporated in the monitor for multiparameter analysis. Software for data acquisition was prepared. A computer set efficient enough to store and transform the acquired data is planned to be purchased in the next stage. A contract for CT examinations in clinical cases within the scientific scope of the project was signed.

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Publications

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MOLECULAR, MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL STUDIES ON MUSCLE AND NERVE IN GENETIC AND ACQUIRED MUSCLE DISORDERS

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In the 2001 the research activity of the Neuromuscular Unit resulted in 12 publications and 4 papers submitted for publication. In addition, 9 research protocols have been prepared.

During the last year, the main emphasis of our work was put on molecular genetics and most members of the Unit participated in molecular research. Many members of the Unit established contacts with molecular centers in Poland and abroad. The topics of our research were the following: spinal muscular atrophy (phenotype-genotype correlation), Emery-Dreifuss dystrophy (proteins of nuclear envelope), facio-scapulo-humeral dystrophy (molecular genetics). The new topic is the genetics of Charcot-Marie-Tooth disease – we are looking for Polish mutations. There is a possibility to join the European bodies coordinating the same projects – namely Eurosmart (spinal muscular atrophy) and Euromen (Emery-Dreifuss dystrophy).

Concerning Duchenne dystrophy – the object of our long-lasting interest – the paper was prepared summarizing the role of dystro-

phin in muscle physiology and pathophysiology, stressing the arguments in favor of and against modern concepts.

Concerning congenital myopathies, we concentrated on desminopathies and congenital myasthenia. The latter is a slow-channel syndrome of acetylcholine receptor (AchR): characteristics of this syndrome involve the ultrastructural features such as abnormal synaptic fissure, mainly in the postsynaptic field.

Two other papers on myasthenia deal with the problem of pregnancy in MG patients.

The electrophysiological studies concern mainly the contribution of neurogenic factor to the myopathy (e.g. Emery-Dreifuss dystrophy, facio-scapulo-humeral dystrophy) and the hyperexcitability of motoneuron. The latter study was performed with cases of amyotrophic lateral sclerosis.

The new line of our research is the modelling of motor unit potential and single muscle fiber potential. The present stage permits us to collect an experience on the effect of fiber diameter, fiber density and the distance from the electrode on the potential's shape. These studies required the preparation of a special program - which has been performed.

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THE ROLE OF EMERIN IN THE PATHOGENESIS OF THE CLINICAL PHENOTYPE OF EMERY-DREIFUSS DYSTROPHY WITH SPECIAL ATTENTION TO CARDIAC PATHOLOGY

Supported by the State Committee for Scientific Research: grant # 4P05B 061 18

Project leader: Irena Hausmanowa-Petrusewicz
Contributors: Małgorzata Dorobek, Anna Fidziańska, Irena Niebrój-Dobosz, Katarzyna Rowińska-Marcińska

The main subject is the role of emerin in the pathomechanism of clinical phenotype development in Emery-Dreifuss dystrophy (EDMD), with special emphasis on cardiac failure.

During the last year, the database of EDMD cases was already available. The missing cases transmitted as autosomal dominant trait were collected and examined by neurologists and cardiologists. Using fibroblast culture from those patients, the characteristics of laminins in autosomal dominant form of Emery-Dreifuss dystrophy (AD EDMD) was summarized. Ten patients passed the full cardiologic check-up, two of them received a pacemaker.

SUICIDE MUSCLE CELL PROGRAM. APOPTOSIS IN SPINAL MUSCLE ATROPHY (SMA) TYPE I

Supported by the State Committee for Scientific Research: grant # 4P05E 057 18

Project leader: Anna Fidziańska-Dolot,
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The muscles of 3 infants with the acute fatal form of SMA with exon 7 deletion were analysed in electron microscopy. In all investigated cases, the muscle cell apoptosis at different stages of death was observed. Our electron microscopic studies demonstrate that suicide muscle cell program accompanies the irreversible acute fatal form of SMA.

The paper entitled "Phenomenon of Schwann cell apoptosis in a case of congenital hypomyelinating neuropathy with basal lamina onion bulb formation" presents the sural nerve biopsy of a child with congenital hypomyelinating neuropathy in the light and electron microscopy. The most prominent pathological finding was the appearance of significant death of Schwann cells with apoptotic morphology and basal lamina onion bulb formation. This finding may suggest that abnormal Shwann cells of premyelin fibres are susceptible to death and their disappearance is responsible for empty basal lamina bulb formation.

HEREDITARY MOTOR SENSORY NEUROPATHIES. PHENOTYPE-GENOTYPE CORRELATIONS

Supported by the State Committee for Scientific Research, grant # 6 P05E 058 20

Project leader: Hanna Jędrzejowska
Contributors: Hanna Drac, Andrzej Kochański, Dagmara Kabzińska, Barbara Ryniewicz

We analyzed the coding region of the Connexin 32 gene (Cx32). In four families with Charcot-Marie-Tooth (CMT) type X disease four novel mutations in Cx32 gene were detected.

In two CMT1B families we detected two missense mutations in Myelin Protein Zero gene (MPZ). The Asn(131)Lys mutation was shown to segregate with focally folded myelin phenotype. The second mutation in MPZ gene is associated with a distinct phenotype of CMT 1B disease. Additionally, we performed a molecular genetic analysis in Charcot-Marie-Tooth type 1A disease (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP).

We introduced SSCP/CSGE analysis and RFLP-PCR analysis in screening for point mutations.

FACIO-SCAPULO-HUMERAL MUSCULAR DYSTROPHY. GENOTYPE-PHENOTYPE CORRELATIONS, SOMATIC MOZAICISM AND INTRAFAMILIAL VARIABILITY

Supported by the State Committee for Scientific Research: grant # 6P05B 071 20

Project leader: Małgorzata Dorobek
Contributors: Irena Hausmanowa-Petrusewicz, Anna Fidziańska, Katarzyna Rowińska-Marcińska, Barbara Ryniewicz, Dagmara Kabzińska

Fifty families with facio-scapulo-humeral muscular dystrophy (FSHD) were collected. DNA of the patients and the available members of the families was isolated and stored. Basic database of the families with FSHD was prepared. The protocols for the follow-up visits (including EMG, evoked potentials, muscle testing) were prepared - according to which the patients are investigated. Collection of clinical data has been started. Necessary laboratory equipment was bought.

A one month stay in Leiden University Medical Center, allowed us to obtain training in DNA research and diagnostic methods applied in FSHD. Molecular analysis performed at the University in Leiden (with dr. S.M. Maarel`s research group), has permitted us to confirm FSHD clinical diagnosis in 10 families and to exclude this diagnosis in one family. This visit has allowed us to start a new

research cooperation in the field of FSHD – we were kindly provided with necessary molecular probes: P13E-11 and 9B6A.

In our research, we applied digestion of genomic DNA with three restriction enzymes (EcoRI, BlnI, XapI), followed by linear gel electrophoresis, Southern blot and hybridization with P13E-11 molecular probe. We have been able to obtain the preliminary results confirming clinical FSHD diagnosis in another seven families.

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PRO- AND ANTI-INFLAMMATORY FACTORS AND IMMUNOLOGICAL MARKERS IN DEMYELINATING DISEASES WITH SPECIAL REFERENCE TO THE EFFECT OF IMMUNOMODULATORY THERAPY

Project leader: Mieczysław Wender
Contributors: Jacek Losy, Grażyna Michałowska-Wender, Elżbieta Tokarz-Kupczyk

Interleukin-18 (IL-18), known also as interferon-gamma (INF-γ) inducing factor is a novel proinflammatory cytokine, which plays an important role in Th-1 response through its ability to induce INF-gamma production in T cells and NK cells. Th-1 immunological response and interferon-γ synthesis are prominent features in multiple sclerosis (MS). It is therefore reasonable to assume that Il-18 may have an important role in the immunopathogenesis of MS. The purpose of our study was to measure Il-18 levels in serum and CSF of 21 patients with the relapsing-remitting form of MS; 9 with active gadolinium enhancing lesions in magnetic resonance imaging (MRI) and 12 without enhancing lesions.

Our results revealed a high statistically significant increase of Il-18 in patients with MS, in both CSF and sera in comparison with the control group. The concentration of Il-18 in patients with MRI gadolinium enhancing lesions were also significantly higher in comparison with patients without active MRI lesions. The results suggest the involvement of Il-18 in the immunopathogenesis of MS, especially in the active stage of the disease.

Among 21 MS patients, 9 had gadolinium enhancing lesions in MRI, whereas more of our patients showed an abnormal albumin ratio. This difference, in our opinion, may be only explained by the

different sensitivity of these two procedures indicating a breakdown of the blood-brain barrier.

It may be speculated that Il-18 can be the target for future therapies in MS by the application of neutralizing antibodies or Il-18 soluble receptor.

Glatiramer acetate (Copaxone) is a synthetic copolymer, know to reduce exacerbation rates in patients with relapsing-remitting MS. Among possible immunological mechanisms of glatiramer acetate (GA) action is the up-regulation of proinflammatory cytokines. However, till now several aspects of GA action on MS are unclear. We present data showing the Il-18 immune response during therapy with Copaxone in patients with relapsing-remitting MS. The patients were receiving GA in a dose of 20 mg daily subcutaneously. Sera were taken before and after 6 months of therapy. In our studies after 6 months of therapy with GA a statistically significant decrease of Il-18 expression in sera of treated MS patients has been observed. The expression of Il-18 after therapy did not differ from the control values.

Interleukin 18 is a potent interferon-gamma inducing factor. Therefore, the significant down-regulation of Il-18, resulted from GA-treatment seems to be one of crucial points in immunomodulatory action of Copaxone in MS patients.

The anti- and proinflammatory cytokines and chemokines contribute to pathophysiological mechanisms in demyelinating diseases. However, it is the subject of debate whether the reactions are specific or connected with general pathological processes in the brain. We have addressed this issue by studying the cytokines in ischemic stroke. The purpose of start studies was to analyse CSF and serum levels of TNFα in patients with ischemic stroke within the 24-hours after the onset of symptoms and to correlate these levels with those of the neurological deficit. We have found significantly increased TNFα levels both in CSF and in serum in stroke patients as compared with controls. The TNFα also were significantly correlated with the neurological impairment 24-hours after the onset of symptoms. The studies suggest the overproduction of TNFα during the first hours of stroke.

In the further work on the subject, the monocyte chemoattractant protein-1 (MCP-1) level in sera and cerebrospinal fluid of ischemic stroke patients was studied 24 hours after the onset of neurological symptoms. Our results demonstrate a significant increase of the cerebrospinal fluid MCP-1 level. The serum level of MCP-1 did not differ from that of control patients. The findings suggest that monocyte chemoattractant protein-1 may play a key role in the inflammatory reaction during the early phase of ischemic stroke.

The studies should be continued to compare the reaction in inflammatory demyelinating diseases and in ischemic stroke.

Collaborating units

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CARDIOVASCULAR, METABOLIC AND NEUROHORMONAL RESPONSES TO SELECTED PHYSIOLOGICAL STIMULI IN HUMAN SUBJECTS IN RELATION TO PHYSICAL ACTIVITY AND DIETARY FACTORS

Project leader: Krystyna Nazar

An influence of body carbohydrate modifications on physiological responses to exercise

Contributors: Tomasz Mikulski, Andrzej W. Ziemba

The aim of this study is to find out whether reduction of body carbohydrate stores and their partial restitution by a high-carbohydrate meal affect metabolic and neurohormonal responses to graded incremental exercise until volitional exhaustion. The investigation was performed in 15 young healthy volunteers who took part in three exercise tests at one-week intervals: (1) after an overnight fasting, (2) 2 hrs after a high-carbohydrate or (3) after a low-carbohydrate meal given following depletion of muscle and liver glycogen by prolonged exercise performed on the preceding day and followed by a 20 hours fasting. During each test, heart rate (HR), O_2 uptake and CO_2 production were continuously recorded, and blood concentrations of lactate [LA], glucose, FFA, leptin, catecholamines, insulin, cortisol, growth hormone and testosterone were determined. The results obtained so far did not show significant effects of carbohydrate store modifications on maximal O_2 uptake and HR, while respiratory exchange ratio, blood glucose, insulin and [LA] concentrations reached significantly higher values during exercise after the high-

carbohydrate meal than during the test performed after an overnight fast or the low-carbohydrate meal. Plasma leptin concentration was significantly lowered by carbohydrate depletion but neither the meal nor the exercise changed the level of this hormone. Further analyses are in progress.

Effects of three day head down bed rest (HDBR) before and after endurance training

Contributors: Hanna Kaciuba-Uściłko, Tomasz Mikulski, Andrzej W. Ziemba, Gerard Cybulski, Wiktor Niewiadomski, Krzysztof Krzemiński

Our previous study (Smorawiński et al. *J Physiol Pharmacol* 2000, 51: 279-289, Smorawiński et al. *J Appl Physiol* 2001, 91:249-251) indicated that the effects of a 3-day bed rest on several responses to physiological stimuli are more pronounced in endurance athletes than in sedentary men. In order to find out whether it was due to the training *per se* a new series of investigations was undertaken in subjects who were submitted to 3-day HDBR before and after 6 weeks of intensive endurance training. All the subjects were living at the students' campus, had the same diet and uniform daily regime of physical activity. Before and after HDBR, blood samples were collected for the measurement of plasma catecholamines, adrenomedullin, cortisol, ACTH, insulin, leptin, TSH and testosterone. In addition, exercise capacity and orthostatic tolerance along with physiological and neurohormonal responses to incremental exercise and to a lower body negative pressure (LBNP) test were determined. Twelve subjects were so far examined. The whole study consisting of 24 subjects will be completed in 2002.

Collaborating units

Department of Sport Medicine, Academy of Physical Education, Poznań, Poland

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Resting metabolic rate, metabolic and hormonal responses to oral glucose load in adolescent girls of different level of physical activity

Contributors: Renata Zabielska, Andrzej W. Ziemba, Tomasz Mikulski

Resting metabolic rate (RMR), thermogenic effect of glucose (TEG) and changes in blood glucose, plasma insulin and catecholamines after glucose ingestion (75 g) were compared in 12 ballet school students and 13 sedentary girls aged 15-18 years. The data show that the two groups have similar RMR and blood glucose levels but the ballet school students respond to glucose load with a significantly greater TEG, lower increases in plasma insulin concentration and higher increases in plasma adrenaline. Therefore, the results did not confirm the literature data suggesting that the high level of physical activity decreases RMR, but they did show that regularly performed exercise leads to increased postprandial thermogenesis due most probably to increased insulin sensitivity and adrenaline secretion.

Effects of aerobic training with respiratory muscle exercise in overweight patients

Contributor: Barbara Kruk

Twenty four men (BMI = 28.9 ± 0.5 kg × m^2) participated in recreational training (30 min walking, 7 days per week) for one month. Then they were assigned to three groups: group 1 – the same kind of training was continued for further two months, group 2 – the subjects additionally performed deep breathing exercise of the yoga type, and group 3 – expiratory muscle training was added. Regular walking decreased heart rate and oxygen uptake during submaximal treadmill exercise, improved glucose tolerance and increased maximal voluntary ventilation. Walking training with the addition of yoga exercise had a beneficial effect on several respiratory indices measured at rest and during exercise. Less pronounced changes were found

after the addition of expiratory muscle training. The results demonstrate that moderate aerobic training supplemented with yoga deep breathing exercise could be recommended for overweight patients.

Collaborating unit

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Effect of exercise on plasma adrenomedullin in men

Contributors: Krzysztof Krzemiński, Barbara Kruk, Tomasz Mikulski

Plasma concentrations of adrenomedullin (ADM) and catecholamines were measured during graded incremental exercise until volitional exhaustion in 10 healthy young men. It was found that during exercise plasma ADM concentration decreases slightly but significantly. Significant correlation was ascertained between changes in plasma ADM and diastolic blood pressure but no relationship was found between changes in plasma ADM and catecholamines. The results suggest that the exercise-induced decrease in plasma ADM is associated with a fall in vascular peripheral resistance.

The effect of α-adrenergic receptor blockade on plasma ADM secretion during static handgrip was studied in patients with heart failure. It was found that both resting and exercise plasma ADM concentrations were diminished by α-adrenergic blockade. Thus, it can be speculated that noradrenaline acting *via* a receptors stimulates ADM secretion.

Collaborating unit

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TRIACYLGLYCEROL SYNTHESIS IN RAT SKELETAL AND HEART MUSCLES – THE CONTROL MECHANISMS

Project leader: Ewa Żernicka

Effects of cold exposure on fatty acid incorporation into skeletal muscle acylglycerols in rats

Contributors: Marcin Synak, Monika Górecka

Skeletal muscle fatty acid uptake after a short term cold exposure was investigated. ^{3}H-palmitic acid incorporation into intracellular acylglycerol stores in skeletal muscles of various fibre types of rats exposed to 6^{0}C for 12 h was measured using the hind-limb perfusion technique. Cold exposure led to an elevation of serum fatty acid levels to 0.387 +/- 0.058 mM ($p<0.05$) as compared with the control value of 0.249 +/- 0.067 mM. Fatty acid incorporation into the intramuscular acylglycerol pool in cold exposed rats remained unchanged as compared with controls in all muscle types examined. The data suggest that although shivering thermogenesis promotes the use of fatty acids as energy substrates it does not alter the fatty acid incorporation into intramuscular acylglycerol stores. It can not be excluded that fatty acid oxidation was accelerated which could not be detected using the perfusion technique applied in this study.

Effects of cold exposure on indices of lipid metabolism in the rat skeletal muscles and myocardium

Contributors: Monika Górecka, Zofia Brzezińska

The dynamics of changes in the indices of lipid metabolism in the rat skeletal muscle and the heart during the first 24 h of cold exposure (6^{0}C) were investigated. Already after 3 h of cold exposure a marked decrease in plasma triacylglycerols (TG) and an increase (by approx.50%) in the plasma free fatty acid (FFA) concentrations were found. These changes were accompanied by increased activi-

ty of the extracellular, active fraction of lipoprotein lipase (LPL) in the myocardium and the soleus muscle. The increase in activity of the intracellular LPL fraction was noted after 6 h in the cold. The highest LPL activity (both fractions) was found after 12 h of cold exposure. These results confirmed our previous data showing a marked increase in the muscle potential for lipid utilization after 24 h of cold exposure and demonstrated that most of the changes occurred within 6 h in the cold. Determinations of mRNA for LPL and uncoupling protein UCP 3 in the muscles are in progress.

Collaborating unit

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DETERMINATION OF THE DEGREE OF RESPIRATORY ARRHYTHMIA ASYMMETRY AS A POTENTIAL WAY OF ESTIMATION OF VAGAL ACTIVITY IN MAN

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Project leader: Wiktor Niewiadomski
Contributors: Krystyna Nazar, Gerard Cybulski, Anna Gąsiorowska, Alicja Kodrzycka

The aim of the study was to find out whether duration of the phase of RR interval lengthening of respiratory arrhythmia during slow breathing (6/min) could be used as an index of vagal activity. Based on previous studies it was hypothesized that this phase could be prolonged when vagal tone during the supine rest would be lowered by standing or performing static exercise. However, using the respiratory signal as a reference, it was found, in 30 young, healthy women and men, that in these three conditions i.e. during slow breathing at: supine rest, static exercise performed in supine position, and at standing, lengthening of RR intervals occurred always at the beginning of expiration and encompassed 1-3 RR intervals. At lower

levels of vagal activity the maximal increase of RR interval (dRRmax) was attenuated. The attenuation of dRRmax paralleled that of difference between the longest and the shortest RR interval in a cycle of respiratory arrhythmia (ΔRR: amplitude of respiratory arrhythmia) during slow and spontaneous breathing. In supine rest these 3 parameters correlated with tonic vagal activity using pharmacological blockade of parasympathetic system in 14 subjects, with dRRmax showing slightly better correlation than the two other parameters.

The results of this study can be applied in all situations when vagal tone during supine rest in healthy, young persons is determined. They validate the use of 3 indices: maximal increase of RR interval (dRRmax) observed at the beginning of expiration during slow breathing (6/min), difference between the longest and the shortest RR interval in a cycle of respiratory arrhythmia (ΔRR: amplitude of respiratory arrhythmia) during spontaneous and slow breathing. Furthermore, the findings point to the possibility that these parameters can reliably assess the vagal tone in circumstances of attenuated vagal activity. Analysis of changes in RR intervals and arterial blood pressure casts doubts on baroreceptor reflex sensitivity (BRS) determination based on respiratory fluctuation of these signals: BRS should be relate not only to phase of breathing, but also to the time elapsed from the beginning of expiration because in some parts of the respiratory cycle BRS value can reflect not the causal relation between blood pressure and heart rhythm but merely the coincidence between them caused by the common factor i.e. respiration.

Collaborating unit

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Publications

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THE ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN THE CONTROL OF RENAL CORTICAL AND MEDULLARY CIRCULATION IN THE RAT

Project leader: Janusz Sadowski
Contributors: Bożena Bądzyńska, Monika Grzelec-Mojzesowicz, Leszek Dobrowolski, Elżbieta Kompanowska-Jezierska

An appropriate perfusion of the renal medulla with blood is a necessary condition of functional integrity of this zone and of normal excretory and regulatory function of the whole kidney. This is of major physiological and pathophysiological importance as the medulla is widely viewed to have a crucial role in maintaining body fluid homeostasis and in the control of arterial pressure. The intrarenal vasculature can respond to neural and a variety of humoral stimuli with vasodilatation or vasoconstriction, resulting in increased or decreased perfusion of renal tissue, respectively. Thus, a depression of medullary circulation, e.g. following a release of renin and increased generation of angiotensin II (Ang II), could seriously endanger the medullary tissue. On the other hand, evidence has accumulated over the years, indicating that perfusion of the renal medulla is controlled separately, independent of the control of blood flow through the cortex.

The present study was undertaken to examine if changing activity of angiotensin II would affect the renal cortical and medullary circulation in a parallel fashion and thus high hormone activity would, indeed, compromise perfusion and, potentially, the function of the medulla. The focus was on the role of two Ang II receptor types, AT_1 and AT_2.

Effects of Ang II on total renal blood flow (RBF), and cortical and medullary blood flow (CBF, MBF: laser-Doppler flux) were studied in anesthetized rats. Ang II infusion (30 ng kg^{-1} min^{-1} i.v.) decreased RBF 27±2% whereas MBF increased 12±2% (both $p<0.001$). Non-selective blockade of angiotensin II receptors with saralasin (3 µg kg^{-1} min^{-1} i.v.) increased RBF 12±2% and decreased MBF 8±2% ($P<0.001$). Blockade of AT_1 receptors with losartan (10 mg kg^{-1}) increased CBF 10±2% ($P<0.002$) and did not change MBF. Losartan given during Ang II infusion significantly increased RBF (53±7%) and decreased MBF (27±7%). Blockade of AT_2 receptors with PD 123319 (50 µg kg^{-1} min^{-1} i.v.) did not change CBF or MBF. Intramedullary infusion of PD 123319 (10 µg min^{-1}) superimposed on intravenous Ang II infusion did not change RBF but slightly decreased MBF (4±2%, $P<0.05$). In summary, it was seen that in anesthetized surgically prepared rats, exogenous or endogenous Ang II may not depress medullary circulation. In contrast to usual vasoconstriction in the cortex, vasodilatation was observed, possibly related to secondary activation of vasodilator paracrine agents (e.g. prostaglandins, kinins and nitric oxide). It was shown that differential regional responses cannot be simply explained by constriction or relaxation of the vascular smooth muscle as a direct consequence of different activation status of two angiotensin receptor types. The present data suggest very strongly that activation of the renin-angiotensin system, as observed under conditions of arterial hypotension, hypovolemia, sodium deficit or following a variety of stressful stimuli, would probably not compromise perfusion of the renal medulla with blood. This is of major importance as even under normal conditions the medullary tissue has only a small reserve of oxygen supply and, indeed, appears to be functioning on the verge of hypoxia

NEUROHUMORAL CONTROL OF RENAL CIRCULATION AND TUBULAR TRANSPORT: ROLE OF DIFFERENT ISOFORMS OF NO SYNTHASE AND SYMPATHETIC INNERVATION

Supported by the State Committee for Scientific Research: grant # 6PO 5A 05821

Project leader: Janusz Sadowski
Contributor: Agnieszka Walkowska

Renal sympathetic nerve activity (RSNA) exerts a tonic vasoconstrictor action on renal microvasculature, at least within the cortex. On the other hand, nitric oxide (*NO*) generated in the kidney by endothelial and neuronal isoforms of *NO* synthase (eNOS, nNOS) is known to be a vasodilator. RSNA and *NO* have also opposed effects on tubular transport, with the former factor stimulating and the latter inhibiting NaCl reabsorption, respectively.

The present project explores interaction of RSNA and *NO* in anesthetized surgically prepared Wistar rats. In a preliminary series i.v. infusion of L-NAME, a nonselective inhibitor of *NO* synthesis at 10μg/kg/min induced an increase in arterial pressure up to 11%, almost parallel decreases in renal cortical and medullary blood flow (CBF, MBF – laser-Doppler measurements), and a decrease in glomerular filtration rate (measured by inulin clearance). All these changes were statistically significant. There was also a significant decrease in sodium and water excretion. Subsequent denervation of the experimental kidney induced an increase in CBF but no significant change in MBF; this response was similar with that observed in a control group in which denervation was performed without L-NAME pretreatment. These preliminary data indicate that *NO* significantly affects circulation in the renal cortex and medulla and may suggest that mechanisms underlying this control are different in the two zones.

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L-ARGININE SUPPLEMENTATION IN CORONARY ARTERY DISEASE AND IN CONGESTIVE HEART FAILURE

Clinical investigation performed in cooperation with the Department of Cardiology, Postgraduate Medical School, Grochowski Hospital, Warsaw.
Head: Prof. L. Ceremużyński

Clinical studies continued this year include two projects.

The influence of L-arginine and antioxidant vitamins A and C on clinical course of myocardial infarction (MI)

This is a prospective, randomized, placebo controlled study coordinated by the collaborating Department of Cardiology. Primary end points are: cardiac death, serious arrhythmias, symptoms of heart failure, infarct extension. Secondary end points include: mortality (1 and 6 months), electrical instability of the heart, biochemical markers of oxidative stress. The cardiovascular Laboratory is involved in the latter part of the study aimed at evaluating the effects of L-arginine and antioxidant treatment upon serum levels of malondialdehyde, lipid peroxides, reduced sulphydryl groups, catalase and glutathione peroxidase in erytrocytes. Completion of the study is planned for Spring 2002.

Relation between serum triiodothyronine, serum catecholamines and clinical course of MI

Recruitment of patients has been completed, manuscript in preparation. Studies completed include evaluation of the controversial issue of whether ischemia/reperfusion during elective coronary

angioplasty evokes myocardial peroxidative injury. We measured indicators of free radical damage to lipids (free malondialdehyde) and proteins (sulphydryl groups) in coronary sinus blood in the patients undergoing elective angioplasty for isolated stenosis of the proximal left anterior descending coronary artery. Results have shown that elective coronary angioplasty with 60 s balloon inflation is a safe procedure that does not induce peroxidative myocardial injury.

We have also shown that ischemia/reperfusion during coronary angioplasty evokes an immediate increase in plasma activity of matrix degrading metalloproteinase-9. The activity of metalloproteinase-2 and plasma level of NO metabolites were unchanged. We suggest that a rapid release of MMP-9 after balloon inflation may contribute to remodeling and/or protect the vascular wall from post-PTCA thrombosis.

We have contributed to clinical investigation aimed at evaluating plasma activity of estrogen and progesterone in young women with ischemic heart disease.

Collaborating unit

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Publications

Cedro K, Marczak E, Czerwosz L, Herbaczyńska-Cedro K, Ryżyłło W: Elective coronary angioplasty with 60 s balloon inflation does not cause peroxidative injury. *Eur J Clin Invest* 2002, 32, 148-152.

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THE IMMUNE CELLS, CYTOKINES, AND DNA IN BODY FLUIDS AND TISSUES IN RESPONSE TO THE ALLO- AND BACTERIAL ANTIGENS

Project leader: Waldemar L. Olszewski
Contributors: Hanna Gałkowska, Bożenna Interewicz

Tissue fluid and lymph lipoproteins undergo chemical remodeling in the intercellular space in the process of reverse cholesterol transport in a different fashion than in plasma. Lipoproteins participate in the immune processes in the tissue fluid.

The aim of the studies was to investigate the composition and structure of lipoproteins in human skin and activation of their reverse transport from tissues to blood.

The composition of skin lymph lipoproteins was analysed in lymph collected from a leg lymphatic over a period of 6 days. Apolipoproteins were found in the LDL fraction. The lymph ApoA-I molecule was larger than the ApoA-I molecule in plasma. The ApoAII, ApoE, total cholesterol and phospholipids were represented in lymph in higher concentrations than in plasma. The lymph HDL fraction level was higher by 30% than it was calculated from the capillary filtration rate. The role of the HDL fraction in increasing the reverse cholesterol transport remains unclear. The main acceptor of the nonestrified cholesterol is the pre-beta-HDL. Administration of ApoAI/PC to volunteers brought about an increase in lymph and plasma pre-beta-HDL. These results point to the mobilizing role of pre-beta-HDL for the unestrified cholesterol from cells to tissue fluid and lymph. The

obtained data indicate that mobilization of cholesterol can by obtained using pharmacological intervention.

The tissue fluid and lymph create environment for the metabolic processes of the parenchymatous cells and response to the factors penetrating tissues and evoking inflammatory reaction.

To investigate foot skin saphrofite fungi in patients with lymph stasis, penetration of fungi to deep tissues and local reaction to fungi. Lymph stasis creates conditions of an immunologically priviledged place.

Twenty-seven fungal strains were isolated from the foot skin swabs. Only in one case were fungi isolated from the inguinal lymph nodes. This is the first report on lack of penetration of fungi through the lymphedematous skin.

Collaborating units

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IMMUNOREGULATORY ROLE OF HEMOPOIETIC BONE MARROW CELLS IN ALLOGRAFTING

Project leader: Waldemar L. Olszewski
Contributors: Bożenna Interewicz, Marek Durlik

The dendritic cells play a dominant role in recognition and initiation of response to alloantigens. Their responsiveness to the immunosuppressive drugs is not clear.

To investigate the initiation of allogeneic reaction by the dendritic cells and the sensitivity of dendritic cells to the immunosuppressive drugs.

The canine dendritic (veiled, Langerhans cells) cells initiated rejection of allogeneic skin grafts in scid mice. They mobilized mouse bystander macrophages and granulocytes for final destruction of the graft. The dendritic cells were resistant to cyclosporin A. Cyclosporin A did not inhibit dendritic cell-lymphocyte cluster formation and alloantigen presentation. The obtained results explain lack of effectiveness of cyclosporin A in allogeneic skin transplantation.

The posttransplantation microchimerism may decrease the responsiveness of the recipient to donor alloantigens. The question remains unanswered as to whether the cellular or genetic material microchimerism is important in attenuating the response to donor antigens

To search for donor free DNA in recipient tissues.

Donor graft DNA was detected in the lymphoid and nonlymphoid tissues of the recipient. Naked DNA injected intravenously did not accumulate in recipient tissues. These results suggest that donor DNA was incorporated into recipient cells as fragments of nuclei. Further studies are carried out in order to identify the cells specifically incorporating graft cellular debris.

The adjustment of an allograft to the recipient, resulting in low level reactivity against donor antigens, may be the result of replacement of donor graft cells by recipient genotype cells.

To study the genotypes of muscle and endothelial cells in aortic grafts after desquamation of donor endothelial cells.

The endothelial and muscular cells of aorta were partly replaced by cells recipient origin. The type of precursor cells is under study.

Collaborating units

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Department of Internal Medicine, Medical School, Warsaw, Poland

THE HUMORAL AND CELLULAR DEFICIT IN PROTRACTED WOUND HEALING AND AFTER TISSUE TRAUMA

Project leader: Waldemar L. Olszewski
Contributors: Robert Słotwiński, Hanna Gałkowska

The local inflammatory reaction is mediated by cytokines, chemokines and growth hormones. The question arises how is the local signal transferred with lymph to the regional lymph nodes.

To investigate the lymphatic system in limbs and the systemic cytokine reaction after tissue mechanical trauma.

Mechanical trauma of limbs does not cause interruption of the lymphatic pathways. However, dilatation of lymphatics and inguinal lymph nodes can be observed in all cases irrespective whether the patients suffered from the bone fracture or only soft tissue injury. Although the healing process was still in active stage, no hypercytokinemia could be observed in peripheral blood.

Early infectious complications after colorectal surgery are not uncommon. The main problem is an early diagnosis of dehiscence of the anastomosis, before sepsis develops.

To investigate which serum cytokines increase early after the development of local inflammation in the peritoneum.

The TNFRI (tumor necrosis factor receptor I) was found to reach high serum levels already on day 1-3, whereas the concentrations of other cytokines rose on day 7. Daily measurement of the TNFRI level may be helpful for early clinical diagnosis of complications of the anastomosis.

Collaborating units

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POSTTRAUMATIC EDEMA OF LOWER EXTREMITIES. PATHOMECHANISM, NEW DIAGNOSTIC MEASURES, TREATMENT TRIALS

Supported by the State Committee for Scientific Research: grant # 4PO5C/047/19

Project leader: Waldemar L. Olszewski
Contributor: Grzegorz Szczęsny

The study was aimed at the investigations of the pathomechanism of development of posttraumatic swellings after open and closed injuries of lower extremities, including bone fractures. In the experimental part, activation of cytokines in tissue fluid and serum, identification of cells participating in the local inflammatory process and release of TGF alpha from the damaged bones were investigated. Enlargement of lymph nodes and increase of class II positive cells were found in the nodes. Bone fracture produced more changes in node cellularity than trauma of soft tissues or an open wound. Clinical evaluation of patients based on measuring acute phase protein levels and analysis of lymphoscintigraphic recordings was carried out. Three months after injury levels of the inflammatory cytokines were back within normal limits, although major changes were seen on lymphoscintigrammes in the lymphatic system of limbs. Enlargement of lymph nodes and dilatation of afferent lymphatics were seen in all cases. There were no differences in the dilatation and enlargemen indexes between patients with bone fractures, soft tissue injuries, thrombotic complications and surgical interventions. The reaction to self-antigens is suggested as the reason for the changes in lymphatics and nodes.

DIABETIC FOOT ULCER – IMMUNOHISTOCHEMICAL ANALYSIS OF INFLAMMATION, ROLE OF BACTERIAL INFECTION, THERAPY WITH ANTIBIOTICS COMBINED WITH CYTOKINES REGULATING WOUND HEALING

Supported by the State Committee for Scientific Research: grant # 4P05B 02316

Project leader: Hanna Gałkowska
Contributors: Waldemar L. Olszewski, Joanna Mijal

We continued the immunohistochemical studies of skin biopsies from patients with diabetic foot ulcers and control orthopedic patients without diabetes. There was reduced expression of the proinflammatory TGFbeta1 at the edge of ulcers in the endothelium and increased expression of type 1 receptor for TGFß1 (67% and 90% of specimens,respectively), compared to the control skin. In the ulcer granulation tissue macrophages expressed either TGFß1 or anti-inflammatory IL10 (42% and 50% of specimens, respectively). In the endothelial cells a reduced expression of the angiogenic factors: VEGF (44% of specimens), EGF, FGF2 and increased expression of GMCSF was observed in all diabetic patients. There was the reduced expression of iNOS and increased expression of eNOS (55% and 50% of specimens). Induced NO synthase (iNOS) is a dominating form in inflammation. Lack of iNOS can reduce the level of endothelial NO, which is a regulatory factor in angiogenesis and vasodilatation. Constitutive NO synthase (eNOS) can reach lower levels of NO than iNOS. Observed by us lack of inflammatory cells and reduced expression of the proinflammatory TGFß1 and angiogenic factors can limit the formation of granulation tissue and subsequently healing of the diabetic foot ulcers.

Collaborating units

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ARTERIAL ATHEROSCLEROSIS: INFLAMMATORY CHANGES IN ARTERIES; CORRELATION WITH CLINICAL SYMPTOMS; PHARMACOLOGICAL CONTROL

Supported by the State Committee for Scientific Research: grant # 6P05D/049/21

Project leader: Bożenna Interewicz
Contributors: Waldemar L. Olszewski, Hanna Gałkowska, Marek Durlik

Atherosclerotic changes in arteries are characterized by infiltration of inflammatory cells under the plaques. The type of infiltrating cells and level of their activation are not well known. This is due to lack of *in vivo* obtainable material from peripheral and coronary arteries. The main unsolved problem is the mechanism of detachment of the plaque.

To characterize the infiltrating cell populations under the atheromatic plaque.

The preliminary studies revealed presence of scavenger CD36 type of cell specifically located under the plaque, in the vicinity of calcium deposition.

THE MECHANISM OF LIVER REGENERATION AFTER PARTIAL HEPATECTOMY IN PATIENTS WITH BENIGN AND MALIGNANT LIVER TUMORS

Supported by the State Commitee for Scientific Research: grant # 6 P05C 035 21

Project leader: Barbara Łukomska
Contributor: Joanna Dłużniewska

Liver constitutes a conditional renewal system in which parenchymal and nonparenchymal cells may be induced to proliferate following toxic damage, hepatitis and surgical resection that culminates in the rapid restoration of hepatic tissue. While considerable interest has been focused on liver restoration rate in patients with chronic hepatitis and cirrhosis, no data have been reported regarding the

regenerating process in liver tumor-bearing patients after liver resection.

The aim of the present study was to determine the biochemical markers for liver regeneration in patients with benign and malignant liver tumors.

Twenty five patients undergoing partial hepatectomy for benign liver tumors and metachronous colorectal carcinoma metastases were studied. AFP values in patients with benign liver tumors and those with colorectal liver metastases were within the reference range in the pre- and post-operative periods. No increase in AFP concentration was noticed after partial hepatectomy in both groups of patients, however, the mean concentration of AFP in malignant liver tumor patients was statistically higher compared with benign liver tumor patients before and 7 days after partial hepatectomy. GGT level showed an increase on day 7 after liver surgery compared to the pre-operative GGT level in both groups of patients. The mean values for serum GGT were significantly higher in colorectal liver metastatic patients than in benign liver tumor patients. It seems that serum concentration of GGT provides sensitive assesment of liver regeneration.

Collaborating unit

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INTERACTION OF OPIOIDS WITH OTHER NEUROPEPTIDE REGULATORY SYSTEMS IN PAIN SIGNAL MODULATION

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Analgesics, like other drugs are constructed to target particular receptor(s) in particular neurostructures of the human body. Recent study have shown that receptors for various analgesics are located not only in structures which regulate nociceptive signals, but also in another structures connected with other functions of the organism as a whole. Activation (or inhibition) of such receptors induce side effects, which often produce a negative component of therapy, like respiratory depression, immunosupression, dependency, et cetera. These ruin hopes for discovery of "ideal" drugs free from unwanted side effects. Recently popular "multidrug therapies" may provide some resolution also to the problem of unwanted side effects. This approach is based on the idea that two drugs with different pharmacological profile will interact positively (additive or synergic effect) on wanted pathways. In consequence, the need for each drug will be lowered and unwanted side effects will be less visible. Nevertheless, multidrug therapy provides number of complications, from simple practical problems with accuracy of daily dosage of several drugs, to pharmacological problems related to different pharmacokinetic and pharmacodynamic profile of each drug. One of solutions to this problem is development of drugs with broad spectrum of complementary

actions (multitarget drugs). Effect of interaction of biphalin, opioid ligand with broad opioid receptor affinity, with ketamine, NMDA antagonist, has been main subject of 2001 year study. We were able to evidence the strong synergic interaction of opioid and NMDA systems on spinal level.

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Publications

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EXPRESSION AND FUNCTION OF THYROID HORMONE RECEPTORS (TR) IN HUMAN CANCERS

Contributors: Monika Puzianowska-Kuźnicka, Agnieszka Madej-Pilarczyk, Agnieszka Krystyniak, Janusz Nauman

Thyroid hormone receptors (TRs) are transcription factors involved in the regulation of cell proliferation, differentiation and apoptosis. TRs are cellular homologs of transcriptionally inactive viral oncogene v-ErbA indicated in neogenesis of avian erythroblastosis, some sarcomas, liver cancer and thyroid abnormalities. Therefore, we checked the hypothesis that the function of TRs could be impaired in human cancers as a result of disturbed expression of *TR* gene and/or its somatic mutations.

Papillary thyroid cancer (PTC)

We have previously shown that the mean expression of *TRA* and *TRB* genes on mRNA level in PTC was significantly lower in cancer tissues in comparison to healthy thyroids and nonmalignant overgrowth – thyroid adenomas. We have also shown that the mean amount of TRß1 protein was significantly higher in tumor tissues than in healthy controls while the increased mean amount of TRα1 protein was statistically not significant. By RT-PCR method cDNAs of TRα1 and TRß1 receptors were cloned from 16 PTCs and sequenced. 100% of analyzed PTCs contained mutated TRß1 gene. In 93.75% of analyzed PTCs mutations resulted in amino acid substitutions. The number of mutations within single clone ranged from

1 to 5. 68.75% of PTCs had TRα1 mutated. In 62.25% PTC cases mutations resulted in amino acid substitution. The number of mutations within single clone ranged from 1 to 6. Interestingly, 30% of identified TRα1 mutants had the same residues mutated (S183N, H184Q, R228H). Analysis of the predicted amino acid sequence of TRα1 and TRß1 showed that amino acid substitutions resulting from gene mutations were distributed throughout the whole length of the protein. Analysis of the transactivation activity of mutant TRs revealed that two mutants containing silent mutations within their cDNAs activated transcription similarly to their respective wild type receptors. All TRα1 and TRß1 mutants with amino acid substitutions were defective in transcription activation (2 - 50% of wild type TR activity). All but two mutants presented dominant-negative activity. In contrast, sequencing of TR cDNAs cloned from healthy thyroid lobes (opposite to the cancers with confirmed TR mutations) revealed no TR mutations. Sequencing of TR cDNAs cloned from thyroid adenomas revealed an intermediate number of mutations, but vast majority of them was silent (no amino acid substitution in the encoded protein). To summarize, we described the highest incidence of genetic alterations, affecting a factor potentially involved in tumorigenesis of PTC. Basing on our results we conclude that TRs, once mutated, could became functionally similar to v-ErbA oncogene and therefore could contribute to PTC tumorigenesis. It remains to be elucidated, though, if their major role is the initiation of this process or if they act as secondary factors that are responsible for tumor progression and poor clinical outcome of the disease.

Renal clear cell carcinoma (RCCC)

Three TRα1 and 7 TRß1 mutants, all bearing mutations within ligand (T3) binding domains, were cloned from RCCC tissues by RT-PCR method. Sequencing reaction revealed that some of them had additional mutations within other domains. The specificity of the cloning and sequencing reactions was confirmed by analysis of TR receptors cloned from healthy kidneys: all of them were wild type.

Analysis of the DNA binding activity, ligand (T3) binding activity and transactivation activity of TR mutants revealed that all of them were functionally impaired in comparison to their respective wild type controls. In addition, majority of them presented (to a different extent) features of dominant-negative receptors.

Collaborating unit

Department of Biochemistry, Medical Center of Postgraduate Education, Warsaw, Poland

ACTIVATION OF 5' DEIODINASE TYPE I PROMOTER BY MUTATED TRs

Contributors: Monika Puzianowska-Kuźnicka, Agnieszka Krystyniak, Agnieszka Madej-Pilarczyk, Janusz Nauman

5' deiodinase type I (5'DI) is an enzyme that catalyses monodeiodination of thyroxine (T4, a major secretory product of thyroid gland) to triiodothyronine (T3), the most biologically active form of thyroid hormone. Low triiodothyronine syndrome (LTS) is present in many severely ill patients, including these suffering from cancers. It has been shown that Tumor Necrosis Factors and other cytokines might be responsible for this effect. However, 5'DI promoter contains two thyroid hormone responsive elements (TREs). We hypothesized then that mutated, functionally impaired TRs present in kidney cancer tissues (kidney is one of the major organs where monodeiodination of T4 occurs) are unable to activate 5'DI promoter properly. Analysis of activation of 1.5 kb 5'DI promoter hooked up to luciferase reporter gene by TRα1 or TRβ1 mutants (previously cloned from human RCCC) revealed that it was indeed impaired (by 30-60%) while compared to the promoter activation by corresponding wild type TR. At present we are trying to analyze mutant TR binding to 5'DI TREs to check if defective protein-DNA binding is responsible for promoter transactivation impairment.

EXPRESSION OF VITAMIN D RECEPTOR IN RCCC

Contributors: Agnieszka Madej-Pilarczyk, Monika Puzianowska-Kuźnicka, Janusz Nauman

Since multiple mutations within thyroid hormone receptors were found in RCCC, we decided to analyze the expression of other hormonal receptors in this cancer. We selected vitamin D receptor (VDR) due to following reasons: VDR belongs to the same subgroup of nuclear receptors as TRs; kidney is a key organ in vitamin D metabolism; it has been shown previously, that expression of VDR can be disturbed in other cancers; active vitamin D metabolites have been already used as a supplementary treatment of other tumor types. We examined 24 tumors, that were divided into 3 groups according to histological grading: G1 (well differentiated) – 6; G2 (intermediate level of differentiation) – 10; G3 (poorly differentiated) – 8; 24 controls that were excised from the opposite pole of the same kidney, and 7 controls originating from the kidneys without cancer. Independently of tumor grading neither Northern nor Western blotting demonstrated statistically significant differences in mean VDR expression in tumor samples in comparison to both control types. We observed not significant tendency to decreasing mean mRNA VDR level in G3 cancers and also not significant overexpression of mean VDR protein in cancers in comparison to corresponding controls in all three groups: G1, G2 and G3. Electromobility gel retardation assays showed disturbed DNA binding by VDR, present in cancer tissue, that was weaker in 52% of the analyzed tumors, identical in 30% of the cases and stronger only in 18% of the tumors, in comparison to the corresponding healthy controls. D3 binding to VDR was weaker in cancers than in controls in 80% cases. These results indicate that despite unchanged VDR expression in RCCC, there might be mutation in VDR DNA-binding and/or ligand binding domain, which alters its function as a transcription regulator. Besides RCCCs seem to be very heterogeneous on the molecular level. The presence of VDR protein in all RCCC, even belonging to G3 group, allows to discuss

theoretically the possibility of differentiating therapy in this type of cancer with vitamin D analogues.

THE ROLE OF GENETIC FACTORS IN THE DEVELOPMENT AND CLINICAL MANIFESTATION OF GRAVES' DISEASE

Supported by the State Committee for Scientific Research: grant # 4PO5B 13119

Project leader: Tomasz Bednarczuk
Contributor: Janusz Nauman

The aim of this study was to analyze the association of genetic factors with the clinical manifestations of Graves' disease (GD). So far, we analyzed the polymorphism of the cytotoxic T lymphocyte associated antigen-4 (CTLA-4) gene. Polymorphism of CTLA-4 (A/G) in exon 1 at position 49 was analyzed by restriction fragment lenght polymorphism. The frequency of 49G allele was significantly increased in patients with GD compared to healthy controls (47% *vs* 34%, c2 test p=0.004, Odds Ratio OR=1.7). In addition, the frequency of allele 49G (53% *vs* 40%, p=0.03, OR=1.7) and 49 G/G genotype (22% *vs* 14%, p=0.02) were significantly increased in patients with GD with ophthalmopathy compared to patients with GD without eye signs. However there was no correlation between CTLA-4 polymorphism and the severity of Graves' ophthalmopathy. These results suggest that: (i) CTLA-4 49 G allele confers genetic susceptibility to GD; (ii) CTLA-4 49 G allele and G/G genotype are associated with the development of Graves' ophthalmopathy.

Collaborating units

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Laboratory of Molecular Biology, National Cancer Institute, Bethesda, USA

GANGLIOSIDES OF BENIGN THYROID TUMORS AND DIFFERENTIATED THYROID CARCINOMA: INFLUENCE OF IMMUNE RESPONSE, THYREOMETABOLIC STATUS AND TREATMENT

Suported by the State Committee for Scientific Research: grant # 0514/P050/98/15

Project leader: Jacek Kiljański
Contributors: Zbigniew Bartoszewicz, Barbara Czarnocka

Lipid-bound sialic acid (LBSA) and ganglioside content, alterations of sialyltransferases expression and activity were studied in tissue samples from thyroid papillary carcinoma, benign thyroid tumors, Graves' disease goiter and normal thyroid. We also studied humoral response against thyroid cancer gangliosides.

We found that in the papillary thyroid carcinoma LBSA content is significantly higher than in benign tumors and in normal thyroid. Main ganglioside in all samples of thyroid tissue (both: normal and pathologic) was GM3. GM3 and GD3 gangliosides constitute 60-80% of ganglioside content in all analyzed tissues. Ganglioside content in benign tumors was similar to that in normal thyroid. There was an increase of GM3 content in Graves disease and an increase of GM1 content in papillary carcinoma. We did not find previously reported increase of Fuc-GM1 ganglioside expression in thyroid carcinoma. We did not confirm reports of presence of anti-ganglioside autoantibodies is sera of patients with thyroid carcinoma.

We found high expression of sialyltransferases SiaT 1 and SiaT 4 on the mRNA level in patients with Graves' disease. On the other hand, there were no significant changes in thyroid cancer as compared with control tissues. In the group of Graves' disease patients, we found striking differences between mRNA levels for both sialyltransferases in consecutive samples. There were also striking differences between mRNA levels in benign tumors and normal thyroid tissue samples from the same patients.

We found an increase of sialyltransferases activity in Graves' disease and in toxic nodular goiter. In non-toxic nodular goiter, there were striking differences of sialyltransferase activity in consecutive patients.

Publications

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ROLE OF EXTRACELLULAR NUCLEOTIDES AND NUCLEOSIDES IN THE REGULATION OF RENAL FUNCTION

Project leader: Stefan Angielski
Contributors: Mirosława Szczepańska-Konkel, Ludmiła Martyniec, Gabriela Langner, Tadeusz Pawełczyk, Maciej Jankowski, Leszek Kalinowski

Vasomotor activity of diadenosine triphosphate (Ap3A), diadenosine tetraphosphate (Ap4A) and diadenosine pentaphosphate (Ap5A) in renal glomeruli

Recently we have shown, for the first time, that natural purinergic agonists ATP, ADP, UTP and adenosine change intracapillary volume of isolated rats renal glomeruli suggesting the potential role of extracellular nucleotides in the regulation of glomerular dynamics. In the present study, we examined the vasomotor properties of Ap3A, Ap4A and Ap5A in renal glomerular capillaries. The results indicated that both Ap4A and Ap5A (1 pM – 100 μM) induced reduction of glomerular intracapillary space (GIS) by 10 to 18 % of the basal value. The constrictor effect of Ap4A and Ap5A was inhibited by the P2-purinoceptors antagonist suramin (10 μM) and was unchanged by P1-purinoceptors antagonist theophylline or DPCPX (1 μM each). In the presence of 1 μM angiotensin II the GIS value was reduced by about 16 % and Ap3A (1 μM) reversed constrictor responses to angiotensin II. The effect of Ap3A was inhibited by suramin but not by DPCPX. The results indicate that in rat renal glomeruli, adenine

dinucleotides act directly – and not through their hydrolysis product adenosine – on P2 receptors through which Ap4A and Ap5A induce contraction of glomeruli and Ap3A relaxation.

Renal hemodynamics response to intravenous infusion of diadenosine tetraphosphate (Ap4A) in anesthetised rat

In the clearance study we compared the effects of intravenous Ap4A with equivalent dose of the reference adenosine precursor NAD on renal function. The role of adenosine in Ap4A and NAD-induced changes was investigated by comparing the effects of these dinucleotides on renal plasma flow (RPF), glomerular filtration rate (GFR) and urinary sodium excretion during i.v. administration of enzyme metabolising adenosine to inosine, adenosine deaminase, and adenosine receptors antagonist theophylline. We observed that both Ap4A and NAD infusions (prime, 1 μmol/kg i.v., followed by 10 nmol/(kg min) each) induced decrease of RPF and GFR, by about 10 and 20%, respectively, and only NAD progressively decreased MABP, maximal to 100 mmHg. In spite of reduction of GFR during Ap4A infusion, the significant increases in sodium and urine excretion occurred: fractional excretion of sodium (FE_{Na}) and urine (FE_{urine}) rose to the 15-fold and 2.5-fold of control value, respectively. In contrast to Ap4A, NAD-induced decrease of GFR was associated with parallel decrease in sodium excretion and urine flow, thus the FE_{Na} and FE_{urine} did not significantly ($p>0.05$) change. Pre-treatment with adenosine deaminase or theophylline reversed inhibitor responses to NAD, whereas Ap4A-induced changes were not affected. The results document that both nucleotides profoundly affect renal function: Ap4A similarly to NAD decreases RPF and GFR, but in contrast to NAD significantly increases urine and sodium excretion.

Regulation of adenosine kinase (AK) gene expression by insulin

Performed experiments revealed that the level of mRNA, enzymatic activity and the adenosine kinase (AK) protein level were de-

creased in kidney, liver and heart of diabetic rats as compared to normal rats. These changes were accompanied by 3.5-fold, 2-fold and 1.2-fold increases of adenosine content in heart, liver and kidneys, respectively. Administration of insulin to diabetic rats restored normal level of adenosine and AK expression. Experiments with simultaneous to insulin administration infusion of 5% glucose revealed that changes in AK expression depended on insulin level and were not related to glucose level. Dependence of AK expression level on insulin were confirmed on cultured podocytes. The level of AK gene transcript increased 60% after addition of 10 nM insulin to the cell culture. Further work with the use of rapamycin (an inhibitor of mTOR/p70S6K), wortmannin (an inhibitor of PI3-kinase) and PD152440 (an inhibitor of MEK) indicated that the insulin regulate expression of AK gene by signalling through the MAP kinase pathway.

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PROMOTIONS

HABILITATION THESIS

Anna Maria Kamińska:
Effect of age and innervation on the rat skeletal muscle regeneration

DOCTOR'S THESES

Agnieszka Marta Bronisz
Regulation of apoptosome by PKC-Raf-1 kinases in N2a neuroblastoma cells

Agnieszka Madej-Pilarczyk
Vitamin D receptor (VDR) expression in human renal clear cell cancer

Paweł Janusz Nauman
Expression of nuclear T3 and retinoid receptors in gliomas

Robert Paweł Ostrowski
Effect of coenzyme Q10 on cerebral ischemic alterations induced by endothelins in the rat

Wiesława Pawłowska-Jenerowicz
Coronary heart disease in women - selected aspects of pathogenesis, clinical assessment and diagnostic abilities

ORGANIZATION OF SYMPOSIA AND CONFERENCES

Polish-German working meeting on organization of Stroke Services. Wierzba, April 7, 2001.

2nd International Conference "Frontiers in Opioid Research and Applications". Warsaw, May 28-29, 2001.

International Conference: "Laboratory Animals in Biomedical Research", MRC, Warsaw, June 6-7, 2001. Co-organized by the MRC. Organizer: C. Radzikowski (Wrocław); J.W. Łazarewicz (Warsaw) was a member of the Organizing Committee.

Seminar Poly(ADP-ribose) polymerase-1 mediated stabilization of wild-type p53 protein: importance for signalling of DNA damage or for duration of the p53 response? Warsaw, June 28, 2001

Symposium "Regulation of retina activity and degeneration. Warsaw, July 19, 2001.

21th Annual Meeting of the European Thyroid Association. Warsaw, August 25-29, 2001.

Scientific Educational Conference: The response of peritoneum on gastric and colon cancer. Warsaw, September 13, 2001.

Biological aspects of replantation and transplantation of limbs. Warsaw, September 13, 2001.

Polish-Russian Working Symposium: "Mechanisms of intracellular signal transduction underlying neuronal plasticity under adaptive and pathological conditions". Days of Polish Science in Russia. St Peterbsurg, Koltushi, October 17-18, 2001. Organizers: J.W. Łazarewicz (Warsaw) and M.O. Samoilov (Petersburg).

Inflammation of arteries of lower limb. Warsaw, October 24, 2001.

Inflammatory aspects of atherosclerosis – immunocytochemistry, bacteriology, and treatment with statins. Warsaw, October 24, 2001.

Immune factors in human tissue and lymph. Warsaw, November 7, 2001.

Immune response of lymphatic system to trauma of limbs. Warsaw, November 7, 2001.

Neurosurgical Meeting: Hakim syndrome – Alzheimer's disease – diagnostic problems and treatment. Pułtusk, November 21-24, 2001.

Posttraumatic edema of lower limbs. Warsaw, November 28, 2001.

Parenteral nutrition supports immunity – immunity markers. Warsaw, December 5, 2001.

Days of Neurochemistry – Genetic and molecular basis of central nervous system pathology and therapy. Warsaw, December 7-8, 2001.

GUEST LECTURES

M. Emad Esmat Nour El Din – Theodor Bilharz Institute, Cairo, Egypt
Impairments of liver immunity in schizostomiasis.

J. Gadek Węsierski – Institute fur Tumorbiologie Krebsforschung, University of Vien, Austria
Poly(ADP-ribose) polymerase-1 mediated stabilization of wild-type p 53 protein: importance for signaling of DNA damage or for duration of the p-53 response.

R. Gniadecki – Department of Dermatology, Bispebjerg Hospital, Kopenhaga, Dania
Mechanism of signal transduction by vitamin D receptor.

E.J. Johns – Birmingham University, United Kingdom
Renal nerve stimulation and the activity of NO in the kidney.

J. Gutkowska – Medical University, Montreal, Canada
Participation of brain in hormonal regulation of heart-blood vessels homeostasis.

M.E. Nagui – Theodor Bilharz Institute, Cairo, Egypt
Contemporary opinions on surgery of spleen.

Z. Wszolek – Rochester University, USA
Temporal frontal dementia - FTDP17 – clinical picture.

G. Nikiforovich – Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis, USA
Conformational models of signal transduction in G-protein coupled receptors.

M. Spatz – National Institutes of Health, Bethesda, USA
A role of cerebrovascular endothelium in ischemia and reperfusion.

R.L. Terjung – College of Veterinary Medicine, University of Missouri, Columbia, USA
Colateral vessel remodeling in experimental peripheral arterial insufficiency.

G. Toth – Institute of Biology, Hungarian Academy of Sciences, Hungary
Synthesis of radiolabeled peptides ligands.

S. Zenin – Medical Center, Ministry of Health, Moscow, Russia
The role of the structure of water in biological systems.