Short Thesis for the degree of doctor of philosophy (PhD)

Studying ischemia-reperfusion-mediated receptor expression in vivo using PET radiotracers in various experimental animal models

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Introduction

Positron emission tomography (PET) is a special non-invasive imaging technique used in nuclear medicine, which is suitable for the investigation of various diseases at the cellular, subcellular or even molecular level. It allows the in vivo study of various physiological and pathophysiological processes. The procedure cannot be performed without radiolabeled molecules. Today, studies performed with [^{18}F]FDG represent the mainstream of clinical PET imaging, but other molecules labeled with ^{18}F (e.g. [^{18}F]PSMA-1007) and ^{68}Ga (e.g. [^{68}Ga]Ga-PSMA-11) are also in use. PET is the imaging technique used most in oncology, which is why radiopharmaceuticals suitable for the molecular study of angiogenesis, such as ^{68}Ga-labeled RGD and NGR peptides, are often the focus of interest in preclinical studies. Many serious diseases are caused by dysregulation of angiogenesis. In conditions related to angiogenesis, new blood vessels are formed, which can directly or indirectly negatively affect the function of the given organ. The list of diseases associated with angiogenesis is growing every year, due to the ever-deeper understanding of the pathophysiological processes. Cardiovascular research is no exception to this trend, for example, ischemic heart disease, proliferative diabetic retinopathy or acute lower limb ischemia, since ischemia-reperfusion (I/R) events play a role in the initiation of angiogenic processes, including through the expression of the aminopeptidase N (APN/CD13) molecule.

The use of animal models is one of the most important and widely used experimental tools in research on vascular diseases. So-called clamping methods, such as the posterior vasoconstrictor in proliferative diabetic retinopathy research or the tourniquet in acute lower limb ischemia research, may be suitable for the development of appropriate disease models, with the implementation of the appropriate protocol, which can be the subject of in vivo PET imaging procedures. Through these, the relationship between angiogenic processes and APN/CD13 molecule expression can be better understood, thereby creating opportunities for translation between preclinical research and human clinical applications.

Objective

The use of animal models in vascular disease research is one of the most important and widely used experimental tools. Ligation methods, such as the posterior occipital clamping in proliferative diabetic retinopathy research or the tourniquet in acute lower limb ischemia research, may be suitable for studying ischemia reperfusion mediated receptor expression using in vivo PET imaging techniques, provided that the appropriate protocol is followed.

The main objective of our research is to study ischemia reperfusion mediated receptor expression (APN/CD13) using diagnostic radiopharmaceutical labeled molecules that, as has been proven in oncology research, are enriched with known specificity based on receptor ligand interaction, thus being suitable for studying angiogenic processes in preclinical models of proliferative diabetic retinopathy and acute lower limb ischemia.

The set goals were achieved in the form of two projects:

Project 1:
Study of temporal changes in APN/CD13 expression in vivo in a rat model of ischemia-reperfusion in proliferative diabetic retinopathy model using positron emission tomography.

Project 2:
Study of temporal changes in APN/CD13 expression in vivo in a rat model of acute lower limb ischemia-reperfusion using positron emission tomography.

Through these, the relationships between ischemia reperfusion and the expression of the APN/CD13 molecule can be better understood, thus opening up opportunities for translation between preclinical research and human clinical applications.

Materials and Methods

Radiochemistry

[^{68}Ga]Ga-NOTA-c(NGR)

For radiolabeling, 1000 µL of [^{68}Ga]$GaCl_3$ was added. After adding the precursor (3 mM, 5 µL) (NOTA-c(NGR)), the reaction mixture was incubated for 5 min at 95°C. After the incubation time, the reaction mixture was left to stand at room temperature (20°C) for 3 min, then passed through a column with a loading volume of 30 mg activated before synthesis (activation: 5 mL 96% EtOH, then washing with 10 mL water for injection). The radioactive reaction product bound to the cartridge was purified with 2 mL water for injection (B.BRAUN, Hungary) in order to remove any metal impurities and uncomplexed free ^{68}Ga. [^{68}Ga]Ga-NOTA-c(NGR) was eluted with 0.2 mL of 0.9% sodium chloride solution (Fresenius Kabi, Germany)/96% ethanol (Merck, Germany)/2:1 volume mixture into a 1 mL Eppendorf tube. [^{68}Ga]Ga-NOTA-c(NGR) was then sterile filtered (Cathivex®-GV, Merck, Germany) and distributed, and diluted to reduce the ethanol concentration to below 10% for further studies.

[^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$

A 5mL Eppendorf tube was used as the reaction vessel. After measuring the precursor (3 mM, 5 µL) (NOTA-c(NGR)), the reaction mixture was incubated for 5 minutes at 95°C.
After the incubation time, the reaction mixture was left to stand at room temperature (20°C) for 3 minutes and then passed through a column of OASIS HLB 1 cc 30 mg, activated prior to synthesis. The radioactive reaction product bound to the cartridge was purified with 2 mL water for injection (B.BRAUN, Hungary) in order to remove any metal contaminants and uncomplexed free ^{68}Ga. [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ was eluted with 0.2 mL of 0.9% sodium chloride solution (Fresenius Kabi, Germany)/96% ethanol (Merck, Germany)/2:1 volume mixture into a 1 mL Eppendorf tube. The radiolabeled molecule was then sterile filtered (Cathivex®-GV, Merck, Germany) and dispended.

[^{18}F]FDG

2-[^{18}F]fluoro-2-deoxy-D-glucose was prepared using a GE Tracerlab FX FDG Synthesizer (GE, USA) synthesis panel, which integrates the labeling reaction and chemical purification processes.

As the first step of the process, active [^{18}F]fluorine introduced with enriched water was placed in a vessel designed for this purpose. By starting the synthesis program, the [^{18}F]fluorine was bound by vacuum on the QMA (WATERS, USA) ion exchange column placed on the panel. The bound [^{18}F]fluorine was separated from the QMA ion exchange column by ionic elution with K_2CO_3 (SigmaAldrich, USA) and transferred to the reaction vessel. In order for the substitution in the precursor (ABX, Germany) molecule to take place, [^{18}F]fluorine had to be made soluble in an organic solvent using Kriptofix 2.2.2 (Merck, Germany). Then, the precursor molecule dissolved in anhydrous acetonitrile (SigmaAldrich, USA) was introduced into the reaction vessel, where the substitution took place under controlled conditions (temperature, pressure) and for a suitable time, during which the triflate group of the TATM molecule was replaced by active fluorine. Then, the unwanted organic solvent in the final product was removed from the reactor. After solvent removal, the acetyl groups were hydrolyzed in an acidic medium, and then the aqueous mixture was purified on a column and packing line. The synthesized [^{18}F]FDG solution was transferred through a sterile filter (Millex-GV, SigmaAldrich, USA) into ampoules, which were separated for testing purposes.

MiniPET-II Small Animal PET Scanner

PET examinations were performed using the MiniPET-II small animal PET camera (University of Debrecen, Hungary) in the laboratories of the Radiobiology Research Group of the Department of Nuclear Medicine, Faculty of Medicine, Institute of Medical Imaging, University of Debrecen. During the imaging examinations, appropriate analgesic protocols were used, and the necessary waiting time was passed after the radiopharmaceutical was administered via the lateral tail vein as an i.v. bolus.

In the framework of **Project 1**, the head region was examined. For the examinations, either [^{68}Ga]Ga-NOTA-c(NGR) or [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ radiopharmaceuticals were used, with an activity of 15 MBq. After a 90-minute waiting time after the administration of the radiopharmaceutical, a 20-minute static PET imaging was performed, recording the data in a single 20-minute frame.

During **Project 2**, we studied the lower limb region. In this case, in addition to [^{68}Ga]Ga-NOTA-c(NGR), we also used [^{18}F]FDG radiopharmaceutical, in both cases with an activity of 15 MBq. After the administration, a 60-minute waiting time was used, and then the imaging was also performed with a 20-minute static PET scan, recording the study in a single frame. The data obtained during the study were evaluated using BrainCAD version 1.124 (University of Debrecen, Hungary). The results obtained were determined in the form of SUV (standardized uptake value) (formula 1) and SBR (signal/background ratio) values (formula 2). In each case, the SUVmean values of the contralateral pair of the examined organ (signal) were taken as the background.

$$\text{SUV} = \text{Concentration in VOI (MBq/mL)} \bigg/ \frac{\text{Injected dose (MBq)}}{\text{Weight (g)}}$$

Formula 1: Formula for standardized admission value

$$\text{SBR} = \frac{\text{SUV}_{\text{mean}} \text{ region of interest}}{\text{SUV}_{\text{mean}} \text{ region of reference}}$$

Formula 2: Signal-to-background ratio formula

The animals were unconscious during imaging, and their body temperature was stabilized at a constant temperature of 37°C±0.5°C using a heated examination table.

Experimental Animals

Our experiments were performed on male Fischer-344 rats. The experimental animals were obtained from Animalab Hungary Kft., a company specializing in their breeding and distribution.

The animals were kept and handled in accordance with Government Decree 40/2013. (II.14.) in the animal house of the University of Debrecen, Faculty of General Medicine, Institute of Medical Imaging, Non-independent Department of Nuclear Medicine (permit number: III/6-KÁT/2015.). The projects were registered by the Workplace Animal Experiments Committee of the University of Debrecen under registration numbers 21/2017/DEMÁB and 28/2022/DEMÁB.

The animals were kept on IVC (individually ventilated cage system) stands. The number of hours of illumination was 12. Feeding: VRF-1 (SDS Diets) rodent food was provided ad libitum. Drinking was carried out with tap water ad libitum. The temperature was 22±2 °C, the humidity was 50±10%. As environmental enrichment, we provided the experimental animals with paper nesting material and a hiding place (paper tube).

Project 1: Characteristics of Animals Used in the Experimental Series on Proliferative Diabetic Retinopathy and the Separated Groups

In the experiment, male Fischer-344 rats (n=15) weighing 250-300 grams were used. The animals were divided into two groups: ischemic group and control group.

Ischemic *group (n=10)*

Ischemia and reperfusion were induced in the left eye of the rats (left bulb-I/R) (n=10). The right eye (right bulb) (n=10) did not undergo intervention (non-I/R), so it served as an internal control. After the ischemia, antibiotic eye drops were used to prevent inflammatory processes.

Control group (n=5)

In the case of the control group, the animals did not undergo any intervention (non-I/R). As a subgroup, following the same logic as the ischemic group; left bulbus-non-I/R (n=5) and right bulbus-non-I/R (n=5), were separated. The animals of the control group were kept under the same animal housing conditions as the ischemic group and the same anesthesia and euthanasia protocol was used.

Project 2: Characteristics of Animals Used in the Experimental Series on Acute Lower Limb Ischemia and the Separated Groups

In the experiment, male Fischer-344 rats (n=15) were used. The body weight of the animals was between 250-300 grams. The animals were divided into two groups: ischemic group and control group.

Ischemic group (n=10)

Ischemia and reperfusion were induced in the left hind limb of the rats (left hind limb -I/R) (n=10). The right hind limb (n=10) did not undergo intervention (non-I/R), so it served as an internal control.

Control group (n=5)

In the case of the control group, the animals underwent intervention (anesthesia, tourniquet application), however, ischemia and subsequent reperfusion were not achieved (non-I/R). As a subgroup, following the same logic as the ischemic group; left hind limb -
non-I/R (n=5) and right hind limb -non-I/R (n=5), were separated. The animals of the control group were kept under the same animal husbandry conditions as the ischemic group and the same anesthesia and euthanasia protocol was used.

Analgesia Protocols

Intraoperative analgesia

Inhalation anesthesia (Eickemeyer Isoflo, Germany) was used for the surgeries and imaging studies (PET), 3% isoflurane (Aerrane, Baxter, USA) + 0.4 liters/minute oxygen and 1.2 liters/minute nitrous oxide (Linde, Hungary) for all projects.

Postoperative analgesia

In order to alleviate the short-term moderate pain experienced by the animals due to the ischemia - and the resulting damage -, oral analgesia was used during the experiment. An ibuprofen-containing analgesic (~15 mg/kg/day - 2.5 mL Motrin (Johnson & Johnson, USA) in 500 mL water) was mixed into the animals' drinking bottles. The animals had free access to the liquid.

End of the experiments

After the imaging studies were completed, the experimental animals were euthanized. This was achieved by isoflurane anesthesia, using 3% isoflurane (Aerrane, Baxter, USA) + 0 liters/minute oxygen and 1.2 liters/minute nitrous oxide (Linde, Hungary).

Protocol for Induction of Ischemia-Reperfusion (I/R) Injury

Project 1: Proliferative Diabetic Retinopathy –Rat Model

After general anesthesia, the central retinal artery supplying the left eye of the rats was ligated. The eyelid was pulled back with sutures, oxybuprocaine eye drops (Humacain 4 mg/mL, TEVA, Hungary) were instilled into the eyes of the animals for local anesthesia, and then a specially prepared clamp (a surgical suture loop passed through a polyethylene cannula) was used to ligate the central retinal artery. The loop was placed behind the eyeball, loosely surrounding the optic nerve, central retinal artery, ciliary arteries, and retrobulbar connective tissue. By tightening the loop and pressing the cannula towards the surface of the optic nerve, ischemia could be induced and maintained for the desired period of time (90 minutes). Continuous tension of the loop was ensured by a vascular clamp pulling the surgical suture during the ligation.

The ischemic state was macroscopically checked with a 120 D lens. During the ischemia, the eye was protected against drying out by a carbomer-based eye gel (VIDISIC eye gel, Dr. Gerhard Mann Chem.-pharm. Fabrik GmbH, Germany). Reperfusion of the retinal tissue was achieved by releasing the constricting ischemia after the ischemia, thus by the return of blood through the arteria centralis retinae. To prevent infections, tobramycin-containing eye drops (TOBREX 3 mg/mL eye drops solution, Novartis Hungária Kft., Hungary) were used. Until the animals woke up, the eyes were protected against drying out again by a carbomer-based eye gel (VIDISIC eye gel, Dr. Gerhard Mann Chem.-pharm. Fabrik GmbH, Germany).
The body temperature of the animals during intervetion was recorded via a rectal thermometer, which was stabilized at a constant temperature of 37°C±0.5°C using a heated operating table.

Project 2: Acute Lower Limb Ischemia –Rat Model

After general anesthesia, the left hind limb was disinfected, then an elastic rubber band (tourniquet) was applied at the same height on the proximal thigh, wrapped in a standard manner and tightened with the same clamping force, to create ischemia. This was followed by 120 minutes of ischemia, which was confirmed by pulse oximeter and observation of cyanotic discoloration of the limb. Ischemia was terminated by removing the rubber band.
The body temperature of the animals during intervetion was recorded via a rectal thermometer, which was stabilized at a constant temperature of 37°C±0.5°C using a heated operating table.

Imaging Protocol

Project 1: Proliferative Diabetic Retinopathy

On days 1, 3, 7, and 10 after ocular I/R induction, experimental and control animals were anesthetized and [^{68}Ga]Ga-NOTA-c(NGR) (approximately 15 MBq in 150 µL saline) was injected via the lateral tail vein. Furthermore, on day 10, [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ was administered (approximately 15 MBq in 150 µL saline). In this case, two consecutive PET scans were performed using the two radiopharmaceuticals separately with a four-hour time difference between injections. The physiological body temperature of the anesthetized experimental animals was maintained using a heated camera bed. The distribution of the radiopharmaceutical was determined by in vivo PET imaging (MiniPET-II small animal PET scanner, University of Debrecen, Hungary) 90 minutes after injection (20-minute static PET

scans). After the last PET scan, the animals were anesthetized and the eyes were prepared for further ex vivo studies (western blot, histology).

Project 2: Acute Lower Limb Ischemia

Two consecutive PET scans were performed on days 1, 3, 5, 7 and 10 after hindlimb I/R induction, using two radiopharmaceuticals separately. Experimental and control animals were anesthetized and [^{68}Ga]Ga-NOTA-c(NGR) (10.03±2.69 MBq in 150 μL saline) and four hours later [^{18}F]FDG (11.33±1.71 MBq in 150 μL saline) were injected via the lateral tail vein. The physiological body temperature of the anesthetized experimental animals was maintained using a heated camera bed. The distribution of the radiopharmaceutical was determined by in vivo PET imaging (MiniPET-II small animal PET scanner, University of Debrecen, Hungary) 60 min after the injections (20 min static PET scans). Following the last PET scan, the animals were euthanized and the samples obtained from the hind limb, after appropriate preparation, were subjected to further ex vivo studies (western blot, immunohistochemistry).

Western blot

Western blot studies were performed in connection with Project 1: Proliferative diabetic retinopathy and Project 2: Acute lower limb ischemia. Rat kidney tissue was used as a positive control.

The samples from the autopsy of the experimental animals were placed in Petri dishes, placed on sterile gauze soaked in physiological saline, stored on ice and transported under refrigerated conditions to the Department of Biopharmacy, Faculty of Pharmacy, University of Debrecen. The Western blot study was performed according to the following protocol:

The received tissue samples were immediately frozen in liquid nitrogen and stored at –80 °C until further processing. To prepare the tissue/organ lysate, the samples were suspended and lysed in ice-cold M-PER protein lysis buffer (Thermo Fisher Scientific, USA) supplemented with protease and phosphatase inhibitors (SigmaAldrich, USA). Tissue samples were disrupted using Tissue Ruptor (IKA®-WERKE GmbH, Germany). Protein quantification of cell lysates was performed using Bradford reagent (home-made). All samples were diluted 4× with Laemmli buffer. An equal amount (40 μg) of each protein sample heated at 95 ◦C for 8 min was loaded for running. The lysates were separated by gel electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) based on the molecular mass of the target protein. Precision Plus Protein Dual Color Standard (BioRad Laboratories, USA) was

used as a molecular weight marker. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). To prevent nonspecific binding of antibodies, blocking was performed with 5% milk-TBS-Tween.

The membranes were then incubated with primary antibody (mouse anti-rat APN/CD13, overnight, 4 °C, 1:500 dilution, sc-13536, Santa Cruz Biotechnology Inc., USA). Incubation with primary antibodies was followed by HRP-conjugated anti-mouse IgG secondary antibody (1:2000 dilution, Thermo Fisher Scientific, USA). The signal was detected by chemiluminescence. The intensity of each band was normalized to HPRT (anti-HPRT, 1:1000 HPRT antibody (B-11) mouse monoclonal, sc-393901; Cell Signaling Technology, USA). The ChemiDoc Imaging System (Bio-Rad, USA) was used to visualize the membranes and quantify the intensity of the bands.

Immunohistochemistry (IHC)

Immunohistochemistry studies were performed in connection with Project 2: Acute lower limb ischemia. Rat kidney tissue was used as a positive control. The samples from the autopsy of the experimental animals were placed in Petri dishes, placed on sterile gauze soaked in physiological saline, stored on ice and transported under refrigerated conditions to the University of Debrecen, Faculty of Medicine, Institute of Medical Imaging, Department of Radiology.

The rat lower limb tissue sample (muscle) obtained from the autopsy was frozen using dry ice and petroleum ether, embedded in a cryomatrix. The tissue sample was sectioned 6 micrometers thick using a cryostat. The frozen sections were placed directly on slides, then fixed in 10% buffered neutral formalin for 5 minutes. The fixative was removed by washing with tap water, and then the immunohistochemical reaction was performed according to the kit manufacturer's instructions. To prevent nonspecific binding of antibodies, the tissue sections were treated with a blocking solution. The tissue sections were incubated with APN/CD13 specific primary antibody (IHCeasy, Proteintech Germany). The tissue sections were incubated with a detectable secondary antibody conjugated to a label. The presence of APN/CD13 was visualized with a chromogen coupled to the secondary antibody. The slides were dehydrated and cleared according to the following protocol so that they could be coverslipped. The prepared tissue sections were examined under a microscope (Nikon Eclipse E800, Nikon Corporation, Japan) to determine the location and intensity of APN/CD13 expression.

Histology

Histological examinations were performed in connection with Project 1: Proliferative diabetic retinopathy. After the extermination of the experimental animals (ischemic and control groups) (killing the animals, removing blood and perfusion with 0.9% physiological saline), the right and left bulbs were removed. The samples were transported in Eppendorf tubes, in physiological saline, under refrigerated conditions to the Department of Radiology, Faculty of Medicine, Institute of Medical Imaging, University of Debrecen.

On the day of receipt of the samples, the bulbs were placed in embedding medium (Shandon Cryomatrix®, Thermo Scientific, United Kingdom) and the blocks were formed. This was followed by the preparation of the aluminum sample holder, which was achieved by filling it with isopentane and placing it in dry ice. After reaching the appropriate temperature of the specimen holder, the specimen blocks covered with embedding medium were frozen by dipping them into the cooling mixture. The bulbs surrounded by embedding medium were frozen according to the sagittal physiological plane. 9 μm diameter sections were prepared from the specimens prepared as detailed above using a cryostat at -21°C (Shandon Cryotome E, Thermo Scientific, United Kingdom). The prepared sections were examined under a light microscope (Nikon Eclipse E800, Nikon Corporation, Japan).

Statistical Analysis

The data shown in the graphs are the results of at least three independent measurements, expressed as mean ± SD. T-test, ANOVA and Mann-Whitney U-test were used to determine significance. The significance level was $p \leq 0.05$(*) and $p \leq 0.01$(**). A commercial software package (MedCalc 18.5, MedCalc Software, Mariakerke, Belgium) was used for all statistical analyses.

Results

Radiochemistry

Project 1: Results of the Production of [^{68}Ga]Ga-NOTA-c(NGR) and [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$

The production of [^{68}Ga]Ga-NOTA-c(NGR) (for project 1 and project 2) and [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ molecules was achieved in an orderly, robust manner, with adequate specific activity and excellent radiochemical purity.

Project 2: Results of the Production of [^{18}F]FDG

The preparation of the 2-[^{18}F]fluoro-2-deoxy-D-glucose molecule was carried out in an orderly, robust manner, with adequate radiochemical purity.

Project 1: Proliferative Diabetic Retinopathy –Rat Model

We performed an in vivo longitudinal study of APN/CD13 receptor expression using a rat ischemic model of proliferative diabetic retinopathy. During the experiments, we performed PET imaging with the radiopharmaceutical [^{68}Ga]Ga-NOTA-c(NGR) to analyze the changes in the presence of the receptors over time based on the results obtained.

Qualitative analysis of the PET images did not show [^{68}Ga]Ga-NOTA-c(NGR) accumulation in any of the visual organs of the experimental animals of the control group on any of the study days (left bulbus -non-I/R, right bulbus -non-I/R). In contrast, in the ligated visual organs of the ischemic group, significant radiopharmaceutical accumulation was detectable 3 days after ischemia (left bulbus -I/R). Furthermore, marked radiopharmaceutical uptake was also observed in the unligated eyes of the ischemic group ten days after ischemia (right bulbus -non-I/R).

During the quantitative analysis of decay-corrected PET images, significantly ($p \leq 0.01$) increased [^{68}Ga]Ga-NOTA-c(NGR) uptake was detected in the ligated eyes of the ischemic group (left bulbus -I/R), where SUVmean values were 0.35±0.06, 0.29±0.05 and 0.33±0.04 at 3, 7 and 10 days after ischemia.

At the first time point (1 day after ischemia), there was no significant difference in SUVmean values between I/R-induced and non-I/R-induced eyes (ischemic group left bulb -I/R vs ischemic group right bulb -non-I/R vs control group left bulb -non-I/R and control group right bulb -non-I/R) ($p \leq 0.05$). At 3 and 7 days after ischemia, the [^{68}Ga]Ga-NOTA-c(NGR) uptake in I/R-induced eyes (ischemic group left bulb -I/R) was approximately twice that detected in the control group, and there was no significant difference in radiopharmaceutical accumulation between control and non-I/R-induced eyes of the ischemic group ($p \leq 0.05$). In contrast, increased [^{68}Ga]Ga-NOTA-c(NGR) uptake (SUVmean=0.23±0.06) was detected in the non-ligated eyes of the ischemic group (right bulb -non-I/R) 10 days after ischemia. The SUVmean values of the left (SUVmean=0.11±0.05) and right (SUVmean=0.09±0.02) bulbs of the control group were approximately two times lower compared to the ischemic group.

Results of the In Vivo Longitudinal Assessment of APN/CD13 Receptor Expression Using [^{68}Ga]Ga-NOTA-c(NGR) PET

In vivo [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ PET imaging was used to determine the expression of the αVβ3 integrin receptor. Analysis of SUV-corrected PET images revealed no increased accumulation in the ligated eyes of the ischemic group 10 days after the induced I/R compared to the other groups (ischemic group left bulb -I/R vs right bulb -non-I/R, control group left bulb -non-I/R, right bulb -non-I/R). This observation was confirmed by quantitative SUV analysis, where there was no significant difference in SUVmean values between the ligated (SUVmean=0.61±0.08) and non-ligated (SUVmean=0.57±0.07) groups of the ischemic group (ischemic group left bulb -I/R vs group right bulb -non-I/R), nor between the ischemic and control groups (SUVmean=0.60±0.08) (ischemic group left bulb -I/R vs control group) ($p \leq 0.05$).

Results of the In Vivo Assessment of $\alpha V\beta_3$ Integrin Receptor Expression Using [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ PET

In vivo [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$ PET imaging was used to determine the expression of the αVβ3 integrin receptor. Analysis of SUV-corrected PET images revealed no increased accumulation in the ligated eyes of the ischemic group 10 days after the induced I/R compared to the other groups (ischemic group left bulb -I/R vs right bulb -non-I/R, control group left bulb -non-I/R, right bulb -non-I/R).

This observation was confirmed by quantitative SUV analysis, where there was no significant difference in SUVmean values between the ligated (SUVmean=0.61±0.08) and non-ligated (SUVmean=0.57±0.07) groups of the ischemic group (ischemic group left bulb -I/R vs group right bulb -non-I/R), nor between the ischemic and control groups (SUVmean=0.60±0.08) (ischemic group left bulb -I/R vs control group) ($p \leq 0.05$).

Results of Signal-to-Background Ratio (SBR) Analysis in PET Studies

Based on the evaluation of decay-corrected PET images, signal-to-background ratio (SBR) analysis was performed. Regarding the APN/CD13 specific molecule [^{68}Ga]Ga-NOTA-c(NGR), it was found that the SBR ratio in the ischemic group was significantly higher on days 3 (SBR=2.65±0.65) and 7 (SBR=3.51±0.99) after ischemia, compared to the SBR values of the control group at the same time points ($p \leq 0.05$). Ten days after ischemia, a high SBR value (SBR=2.11±0.95) was also observed in the ischemic group, but this difference was not significant compared to the control group ($p \leq 0.05$). The SBR values of the control group remained unchanged during the study, indicating that there was no specific accumulation of [^{68}Ga]Ga-NOTA-c(NGR) in the studied animals.

With the administration of the αVβ3 integrin receptor specific radiopharmaceutical, [^{68}Ga]Ga-NODAGA-[c(RGD)]$_2$, ten days after ocular I/R, there was no significant difference in SBR values between the ischemic and control groups ($p \leq 0.05$).

Results of Western Blot and Histological Examinations

The expression of APN/CD13 and αvβ3 molecules was examined by Western blotting ten days after ischemia. Rat kidney tissue was used as a positive control for the detection of APN/CD13 expression, as this tissue is known to show high levels of expression. This allowed us to confirm the efficiency and specificity of the Western blotting procedure and served as a basic reference point for the other samples tested.

Analysis of APN/CD13 expression in ocular tissue samples revealed significantly higher levels of APN/CD13 in the ligated bulbs of the ischemic group (left bulb -I/R) than in the unligated eyes of the same animals (right bulb -non-I/R) and in the control group ($p \leq 0.05$). There was no significant difference between the unligated (right bulb -non-I/R) and control group visual organs ($p \leq 0.05$).

Histological examinations demonstrated severe structural damage to the retina in the ganglion cell layer (GCL), inner plexiform layer (IPL), and inner and outer nuclear layers (INL and ONL) in the ligated bulbs of the ischemic group (left bulb -I/R). In the hematoxylin-eosin (HE) stained histological specimens, there was a significant decrease in the number of ganglion cell layer components, indicating cellular disintegration and necrosis. In the inner plexiform layer, there was blurring of the area and loss of physiological structure. Karyolysis (dissolution of the nuclei) and karyopyknosis (shrinkage of the nuclei) were observed among the cells of the inner nuclear layer (INL), which is a sign of severe cellular damage. In addition, the order of the nuclei among the cells of the outer nuclear layer (ONL) was disrupted. A relative increase in extracellular space was detected in the samples, which may be a consequence of the weakening of intercellular connections. In the samples of the control group, the structure and density of the cell layers were normal and did not show any differences. The expression of the αvβ3 integrin receptor was also analyzed in the experimental groups. Rat kidney tissue was also used as a positive control for the detection of the αvβ3 integrin receptor. Based on the results, there was no significant difference in the expression of the αvβ3 receptor between the samples of the ischemic and control groups ($p \leq 0.05$). The level of αvβ3 receptor expression was similar in both ligated and non-ligated eyes and did not show any significant difference compared to the values of the control group ($p \leq 0.05$).

Project 2: Acute Lower Limb Ischemia –Rat Model

Results of the In Vivo Longitudinal Assessment of APN/CD13 Receptor Expression Using [^{68}Ga]Ga-NOTA-c(NGR) PET

We performed an in vivo longitudinal study of APN/CD13 receptor expression using a rat ischemic model of acute lower limb ischemia. During the studies, PET imaging was performed with the radiopharmaceutical [^{68}Ga]Ga-NOTA-c(NGR), with the aim of analyzing the changes in the presence of receptors over time based on the obtained results.

In the experiment, we compared PET imaging results with the radiopharmaceutical [^{68}Ga]Ga-NOTA-c(NGR) in hindlimbs that had undergone I/R (ischemia-reperfusion) injury, compared with non-I/R-induced hindlimbs in the ischemic group, and non-IR hindlimbs in the control group, to explore the effects of ischemia-reperfusion on the APN/CD13 receptor pattern.

Visual assessment revealed that the uptake of I/R hindlimbs (left hindlimb -I/R) in the ischemic group showed a gradual increase from the first day to the seventh day after induction of ischemia, and then a sharp decrease in [^{68}Ga]Ga-NOTA-c(NGR) uptake was observed on the 10th day. In contrast, lower radioactivity was detected in the non-I/R hindlimbs (ischemic and control groups).

In agreement with the visual analysis, it was observed that the SUVmean value in the I/R hindlimbs in the ischemic group (left hindlimb -I/R) continuously increased from the first day (SUVmean: 0.090±0.005) to the seventh day (SUVmean: 0.230±0.015). SUVmean values were highest on day 7 (0.230±0.015), followed by a sharp decrease on day 10 (0.123±0.010). In the ischemic group, significantly increased [^{68}Ga]Ga-NOTA-c(NGR) accumulation was detected in the I/R hindlimbs (left hindlimb -I/R) on the fifth, seventh and tenth days after ischemia induction compared to the normally perfused hindlimbs in the same cohort (right hindlimb -non-I/R), and the SUVmean values were 0.138±0.010, 0.230±0.015 and 0.123±0.010 for the I/R hindlimb on the fifth, seventh and tenth days, and 0.085±0.012, 0.088±0.015 and 0.093±0.011 for the non-I/R hindlimb at the same measurement times ($p \leq 0.05$). The maximum of the longitudinal study was observed on the seventh day for the [^{68}Ga]Ga-NOTA-c(NGR) uptake value.

During the evaluation of the performed studies, no significant difference was detected between the I/R and non-I/R hindlimbs in the animals in terms of SUVmax values (ischemic and control groups) ($p \leq 0.05$). However, on the first and third days after ligation, no significant difference was found between the SUVmean values of the I/R and non-I/R hindlimbs in the ischemic group (SUVmean values: 0.090±0.005 and 0.100±0.007 on the first and third days, and 0.080±0.005 and 0.075±0.006 on the first and third days, respectively; $p \leq 0.05$). Furthermore, at the fifth and seventh day examination times, the I/R hind limbs (left hind limb -I/R) showed nearly 2-3-fold higher [^{68}Ga]Ga-NOTA-c(NGR) uptake than the normally perfused limbs (right hind limb -non-I/R) in the ischemic group. Furthermore, the radiopharmaceutical accumulation in the I/R hindlimbs (SUVmean: 0.138±0.010 (day five) and 0.230±0.015 (day seven)) was approximately two to three times higher than in any leg of the healthy animals of the control group at the same measurement time (left hindlimb - non-I/R SUVmean: 0.070±0.004 (day five) and 0.073±0.008 (day seven); right hindlimb - non-I/R SUVmean: 0.070±0.005 (day five) and 0.071±0.009 (day seven)). In addition, no significant difference was detected in the [^{68}Ga]Ga-NOTA-c(NGR) uptake of the non-I/R hind limbs of the ischemic group and the non-I/R hind limbs of the control group, nor between the left and right non-I/R hind limbs of the control group ($p \leq 0.05$).

Results of the In Vivo Longitudinal Assessment of Metabolism Using [^{18}F]FDG PET

A non-specific in vivo longitudinal study was performed using a rat ischemic model of acute lower limb ischemia. During the studies, PET imaging was performed with the radiopharmaceutical [^{18}F]FDG, with the aim of analyzing the changes in the accumulation of the radiopharmaceutical over time based on the results obtained.

During the studies, quantitative PET data analysis was performed, which showed a continuous increase in the accumulation of [^{18}F]FDG in the left hind limb -I/R limbs of the ischemic group between the 1st (SUVmean: 0.70±0.26) and 7th (SUVmean: 1.18±0.32) examination times, followed by a decrease on the 10th day (SUVmean: 0.59±0.30). This trend is similar to the results of the [^{68}Ga]Ga-NOTA-c(NGR) studies. In addition, on days 3 (SUVmean: 0.90±0.28), 5 (SUVmean: 1.09±0.30) and 7 (SUVmean: 1.18±0.32), the [^{18}F]FDG concentration in the I/R hindlimbs (ischemic group, left hindlimb -ischemic-I/R) was significantly higher than that in the non-I/R hindlimbs of the same group (right hindlimb -non ischemic-non-I/R) and in the non-I/R body parts of the control group (left hindlimb -non ischemic-non-I/R and right hindlimb -non ischemic-non-I/R) measured on the same

experimental days ($p \leq 0.05$); moreover, approximately 2-3 times more radioactivity was detected in the I/R hindlimbs than in the non-I/R hindlimbs of the same animals.
There was no significant difference in metabolic activity between the I/R and non-I/R hindlimbs of the ischemic group on either day 1 or day 10 after induction of ischemia (SUVmean values on day 1 were 0.70±0.26 and 0.65±0.21, and on day 10 were 0.59±0.30 and 0.51±0.17) ($p \leq 0.05$). Similarly, [^{18}F]FDG accumulation in the I/R hindlimbs did not differ from the uptake values in the non-I/R hindlimbs of the control group on days 1 and 10 after the procedure ($p \leq 0.05$).

Results of Western Blot Analysis

The expression of APN/CD13 molecules was analyzed by Western blot in both animal groups. A significantly higher level of APN/CD13 protein expression was detected in the I/R hind limbs of the ischemic group (left hind limb -I/R) compared to the non-I/R hind limbs of the ischemic group and the control group on days 1, 3, 5, 7 and 10 after induction of ischemia ($p \leq 0.05$). Furthermore, there was no significant difference in protein expression between the non-ischemic hind limbs of the ischemic group (right hind limb -non-I/R) and the control group (non-I/R) subgroups. The protein expression showed a continuous increase on days 1, 3, 5, 7 and then a significant decrease was observed on day 10. According to the test results, the maximum expression was determined on the seventh day.

Results of Immunohistochemistry Analysis

Muscle tissue samples from the limbs of the experimental groups were subjected to immunohistochemical analysis. This was done in order to follow the temporal changes of APN/CD13 expression related to ischemia at the molecular level. During the study, the presence of APN/CD13 protein was indicated by brown staining, which provided an opportunity to visualize and qualitatively analyze the changes at the tissue level. In order to compare the receptor pattern of skeletal muscle tissues of the ischemic and control groups, we analyzed the samples separated in the same way as the western blot study, using equivalent logic. During the comparison, the samples were prepared and processed according to the same protocol to ensure the comparability of the results.
On the first day, in the limbs of the ischemic group that underwent ischemia (left hind limb - I/R), weak, scattered brown staining was observed in the muscle tissue of the ischemic limb, which indicated the low level of the protein. By the third day, the intensity of staining was clearly enhanced and the size of the stained areas also increased significantly, indicating an

increase in expression and a larger number of affected cells, and by the seventh day, the brown staining reached its maximum intensity, reflecting the peak of APN/CD13 expression in the ischemic tissues. Subsequently, the intensity of APN/CD13 expression slightly decreased on the 10th day after the intervention, indicating a change in molecular processes.

Barely detectable APN/CD13 positivity was observed in the unligated hind legs of the ischemic group (right hind limb -non-I/R), and similar results were obtained in the normally perfused (non-IR) histological sections of the control group. These non-I/R samples of the control group served as negative controls for the examination of APN/CD13 expression, ensuring the validity and specificity of the results.

Rat kidney tissue was used as a positive control for the detection of APN/CD13 expression, as this tissue is known to show high levels of expression. The reason for its use as described above was to confirm the efficiency and specificity of the immunohistochemical procedure. The intense brown staining detected in the kidney tissue indicated with high certainty the presence of APN/CD13, which served as an essential reference point for the other samples tested.

New scientific results of the dissertation

The following new results were obtained during the course of our research:

- In the first series of experiments, in a rat animal model of proliferative diabetic retinopathy, we demonstrated a correlation between the expression level of the APN/CD13 receptor, which is involved in the angiogenic processes initiated by ischemia-reperfusion, and the SUVmean values of [^{68}Ga]Ga-NOTA-c(NGR) accumulation, using PET imaging.

- A longitudinal study was performed on days 1, 3, 7 and 10 after I/R, where we examined the temporal changes in the accumulation of the radiolabeled molecule.

- Based on these, [^{68}Ga]Ga-NOTA-c(NGR) is a suitable radiotracer for detecting quantitative and temporal changes in the expression of APN/CD13 induced by ischemia-reperfusion in a rat model of diabetic retinopathy.

- In the second series of experiments, in an animal model of acute lower limb ischemia, we demonstrated a correlation between the expression of the APN/CD13 receptor, which is involved in the angiogenic processes initiated by ischemia-reperfusion, and the SUVmean values associated with [^{68}Ga]Ga-NOTA-c(NGR), as well as changes in glucose metabolism and [^{18}F]FDG accumulation, using PET imaging.

- A longitudinal study was performed on days 1, 3, 5, 7 and 10 after I/R, where we examined the temporal changes in the accumulation of radiolabeled molecules.

- We concluded that [^{68}Ga]Ga-NOTA-c(NGR) is a suitable radiotracer for the detection of quantitative and temporal changes in ischemia-reperfusion-induced APN/CD13 expression and [^{18}F]FDG in the rate of glucose metabolism in an animal model of acute lower limb ischemia.

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Publication list

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List of publications related to the dissertation

1. **Farkasinszky, G.**, Péli-Szabó, J., Károlyi, P. K., Rácz, S., Dénes, N., Papp, T., Király, J., Szabó, Z., Kertész, I., Mező, G., Halmos, G., Képes, Z., Trencsényi, G.: In Vivo Imaging of Acute Hindlimb Ischaemia in Rat Model: a Pre-Clinical PET Study.
   Pharmaceutics. 16 (4), 1-14, 2024.
   DOI: http://dx.doi.org/10.3390/pharmaceutics16040542
   IF: 4.9 (2023)
2. **Farkasinszky, G.**, Dénes, N., Rácz, S., Kis, A., Péli-Szabó, J., Opposits, G., Veres, G., Balkay, L., Kertész, I., Mező, G., Hunyadi, J., Trencsényi, G.: In Vivo imaging of Ischemia/Reperfusion-mediated Aminopeptidase N Expression in Surgical Rat Model Using Ga-NOTA-c(NGR).
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   IF: 2.3

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List of other publications

3. Arató, V. Z., Képes, Z., Péli-Szabó, J., **Farkasinszky, G.**, Sass, T., Dénes, N., Kis, A., Opposits, G., Jószai, I., Kálmán, F. K., Hajdu, I., Trencsényi, G., Kertész, I.: Acute Myelomonoblastic Leukemia (My1/De): a Preclinical Rat Model.
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4. Girgis, M. M. F., **Farkasinszky, G.**, Fekete, K., Fekete, I., Vecsernyés, M., Bácskay, I., Horváth, L.: Seriousness and outcomes of reported adverse drug reactions in old and new antiseizure medications: a pharmacovigilance study using EudraVigilance database.
   Front. Pharmacol. 15, 1-17, 2024.
   DOI: http://dx.doi.org/10.3389/fphar.2024.1411134
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