THE FREE RADICALS AS SECOND MESSENGERS AND THEIR PATHOPHYSIOLOGICAL ROLE IN THE CARDIOVASCULAR SYSTEM AND IN THE KIDNEY

Ph.D. theses

(Summary)

Mazák István M.D.

Head of the Ph.D. School: Prof. Dr. Nagy Judit

Supervisor: Prof. Dr. Wittmann István

University of Pécs, Medical Faculty

2nd Department of Medicine and Nephrological Centre

Pécs, 2007

Abbreviations:

AGE	-	advanced glycation endproducts
Ald	-	aldosterone
AP-1	-	activator protein-1
AT_1	-	angiotensin receptor 1
$[Ca^{2+}]_i$	-	free intracellular ionized calcium
cGMP	-	cyclic guanosine monophosphate
CML	-	N^{ε}-carboxymethyl-lyzine
CV	-	cardiovascular
DCF-DA	-	dichlorofluorescein diacetate
DCF	-	dichlorofluorescein
DMSO	-	dimethyl sulfoxide
EGF	-	epidermal growth factor
EGFR	-	epidermal growth factor receptor
EPHESUS	-	Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study
ER	-	endoplasmic reticulum
ERK 1/2	-	extracellular signal-regulated kinase 1/2
ESR	-	electron spin resonance
Fe^{2+}	-	ferrous iron
Fe^{3+}	-	ferric iron
Fura-2-AM	-	fura-2-acetoxymethylester
GSH	-	reduced glutathione
H_2O_2	-	hydrogen peroxide
JNK	-	c-jun N-terminal kinase
MAPK	-	mitogen activated protein kinase
MG	-	methylglyoxal
MR	-	mineralocorticoid receptor
NADH	-	nicotinamide adenine dinucleotide
NADPH	-	nicotinamide adenine dinucleotide phosphate
NF-κB	-	nuclear factor-kappa B
NO	-	nitrogen monoxide
NOS	-	nitrogen monoxide synthase
cNOS	-	constitutive nitrogen monoxide synthase
$O_2^{\bullet}$	-	superoxide free radical
$^{\bullet}OH$	-	hydroxyl free radical
OPA	-	orto-phthalaldehyde
p38	-	p38 kinase
RAAS	-	renin-angiotensin-aldosterone system
RAGE	-	AGE receptor
RALES	-	Randomized Aldactone Evaluation Study
RAS	-	renin-angiotensin system
RBC	-	red blood cells
SB	-	smoke buffer
SD	-	Sprague Dowley
SEM	-	standard error of the mean
SH	-	sulfhydryl group
SOD	-	superoxide dismutase
Spi	-	spironolactone
dTGR	-	double transgenic rat for human renin and human angiotensinogen gene
TNF-α	-	tumor necrosis factor alpha

1. INTRODUCTION

Many endogenous and exogenous factors are important in the pathophysiology of cardiovascular diseases, type 2 diabetes mellitus and kidney diseases. This introduction is a short summary of some of these factors.

1.1. Free radicals

Free radicals are very reactive molecules (superoxide ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), nitrogen monoxide (NO)). Hydrogen peroxide (H_2O_2) and peroxynitrite are powerful oxidizing agents but not free radicals.

Free radicals are produced in the presence of ferrous iron - among others - through the decomposition of H_2O_2 (Fenton reaction). $O_2^{\bullet}$ can be transformed to $OH^{\bullet}$ (Haber-Weiss reaction).

Free radicals, produced in the living organisms – produced for example by the enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase – have important role in the regulation of several physiological processes. They are working as second messengers, for example in the cell proliferation, gene expression and regulation of hormone production. $O_2^{\bullet}$, produced by NADPH oxidase have a crucial role in the regulation of mitogen-activated protein kinase (MAPK) system. Antioxidant systems (superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidase, glutathione reductase) are important in the elimination of free radicals.

1.2. Effect of smoking

Cigarette smoke contains a large amount of radicals and reactive oxygen-derived substances. Free radicals formation is also enhanced in organism by smoking (for example through smoke activated NAD(P)H oxidase). These factores are important in the development of cardiovascular complications induced by cigarette smoke. Smoke potentiates thrombocyte aggregation, monocyte adhesion to the endothelial surface and migration through the endothelial monolayer to the vascular wall.

1.3. Effects of advanced glycation end products

Level of advanced glycation end products (AGE) is increased in the serum and in the tissue of the patients with diabetes mellitus and end stage renal disease. AGEs are produced changing

the properties of the meals with cooking for example The term of carbonyl stress means the accumulation of very reactive carbonyls e.g., 3-deoxyglucosone, methylglyoxal (MG) and glyoxal, which are produced e.g. in the Maillard reactions, in the formation of AGEs. AGEs enhance free radical production – among others – by the binding to their receptors, called RAGE (receptor for AGE).

1.4. Effect of iron

Iron is essential for many enzymes and for the oxygen transport, but it can catalyze harmful processes, as well. In diabetes mellitus is released in a greater amount, causing this way tissue damage. Iron catalyzes free radical production mainly in the form of complexes and enhances AGE production, as well.

1.5. Role of intracellular calcium

Increase of intracellular calcium level can lead to cell damage and cell death. H_2O_2, $O_2^{\bullet}$ and angiotensin II increases an elevation of intracellular ionized calcium level ($[Ca^{2+}]_i$). In the regulation of $[Ca^{2+}]_i$ are implicated cell membrane channels, calcium binding proteins and storages (endoplasmic reticulum, mitochondria, Golgi network).Agonists-induced elevation of $[Ca^{2+}]_i$ activates constitutive nitric oxide synthase (cNOS) enzyme, which produces nitric oxide ($^{\bullet}NO$).

1.6. The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system is important in the regulation of blood pressure, electrolyte-, volume- and acid-base balance. Angiotensin type 1 receptor (AT_1) promotes cell proliferation, regulates the structure of vessel wall and tissues. Aldosterone (Ald) causes Na^+-retention, enhanced potassium excretion, sympathic activation, fibrosis and vessel wall damage. The non-genomic effect (can't be blocked with cycloheximide and actinomycin-D) of Ald was observed on many cell types and tissues. Ald modulate intracellular pH, increases intracellular Na^+ and Ca^{2+}, intracellular volume, epithelial growth factor receptor (EGFR) and extracellular signal-regulated kinase 1/2 (ERK1/2) independently of transcription and translation. The effect of Spi and Epl confirmed that Ald has important role in the pathogenesis of the CV system.

1.7. The MAPK system

ERK1/2, c-jun-N-terminal kinase (JNK), different isoforms of p38 kinase, ERK3 and 4 and ERK5 are members of MAPK family. Among others some cytokines, angiotensin II and integrins lead to the ERK1/2 activation. ERK1/2 activates Elk-1, c-MYC, c-Jun and AP-1 transcription factor, which factors are involved in the regulation of cell proliferation, differentiation and survival. JNK can be activated by cytokines, G-protein coupled receptors and growth factors. JNK is important in the regulation of apoptosis, cell proliferation, differentiation, survival and in the stress response. Phosphorylation of p38 kinase is induced by oxidative stress, cytokines (for example IL-1, TNF-α) and angiotensin II among others. The proliferation of cells was blocked with inhibition of p38 kinase. Effect of p38 kinase is important for platelet aggregation, immunological and inflammatory processes, and cell adhesion.

2. AIMS

Our purpose was to investigate the risk factors and free radicals, which are involved in the pathophysiology of CV system and renal diseases under in vitro and in vivo conditions.

- We have investigated the effect of MG and iron on the free radical formation and calcium accumulation in red blood cells (RBC).
- We have investigated the interaction of MG with arginine and the effect of iron on this reaction.
- We have investigated the redox reaction between iron and MG.
- We have investigated the effect of smoke and one of possible constituent of smoke, formaldehyde, on bradykinin-induced calcium increase in, an in vitro system.
- We have investigated the effect of Ald on MAPK system ad on free radical production under in vitro conditions.
- We have investigated the effect of Ald on the angiotensin II-induced MAPK activity.
- We have investigated the effect of Spi on the angiotensin II-induced MAPK activity and on the angiotensin II-induced free radical production.
- We have investigated the effect of Epl on the blood pressure, cardiac function, cardiac hypertrophy, albuminuria and renal fibrosis in double transgenic rats, harbouring human renin and angiotensinogen gene (dTRG).
- We investigated the effect of protein rich diet on the serum-AGE level, urine-AGE level and renal function in young healthy volunteers.

3. METHODS AND RESULTS

3.1. Effect of MG and iron on free radical production and on $[Ca^{2+}]_i$ concentration in human RBCs

We have investigated the effect of MG on the free radical production in RBCs, with fluorescence method. The intracellular low molecular weight thiol group content was measured in RBCs. The change of the $[Ca^{2+}]_i$ was detected in RBCs with spectrofluorimeter. The effect of MG and ferrous iron (Fe^{2+}) was observed in the presence and absence of scavengers and iron-complexing agents.

RBCs, obtained from healthy volunteers were incubated with dichlorofluorescein diacetate (DCF-DA) and Fura-2-acetoxymethylester (Fura-2-AM). Dichlorofluorescein (DCF) is a fluorescence dye detecting the free radical production and Fura-2 is another fluorescence dye, which indicates the changes of $[Ca^{2+}]_i$. Experiments were done with scavengers (SOD, catalase, trolox, desferrioxamine, GSH), as well.

Mean ± S.D. were calculated from six independent measurements. Student's unpaired *t*-test was used for statistical analysis.

Ferrous iron (100 μM) and MG (1 mM) induced a concentration dependent increase of the free radical production. Both MG and Fe^{2+} increased $[Ca^{2+}]_i$ in RBCs.

The effect of co-incubation of RBCs with ferrous iron plus MG did not differ from the effect of ferrous iron alone, on fluorescence of DCF. The effect of co-incubation was significantly lower than the effect of MG alone in the case of calcium accumulation.

All the antioxidants and desferrioxamine used in our experiments significantly decreased both the Fe^{2+} and the MG induced oxidative stress and calcium accumulation in RBCs.

A fluorescence method, using *O*-phthaldialdehyde (OPA) was applied for the determination of low molecular weight thiol group (SH-group) levels. Fluorescence was read at 340 nm excitation and at 425 nm emission wavelengths before and after the addition of 10 mg/ml OPA in methanol. Statistical significance was verified with Mann-Whitney test.

The low molecular weight SH-group level was decreased by 46-66%, after incubation of cells with MG for 5-30 minute (significant decrease after 10 to 30 min of incubation ($p < 0.05$)).

3.2. Effect of iron on free radicals derived from the co-incubation of arginine and MG

To investigate the catalyzing effect of iron, Fe^{2+} and Fe^{3+} was added to the solution containing MG and arginine. Desferrioxamine (10 mM) and ferrozine (40 mM) were used for the chelation of different forms of iron. The electron spin resonance (ESR) measurements were carried out under alkaline pH (8.5). The ESR settings were as follows: microwave power: 20 mW, modulation amplitude: 0.2 G, scan width: 100 G.

The interaction between arginine and MG gives an ESR spectrum which was similar to the spectrum showed by Yim and co-workers in the case of alanine+MG interaction. The Fe^{3+} increases and Fe^{2+} decreases the ESR spectrum intensity.

Fe^{3+} evoked a concentration dependent increase and Fe^{2+} a concentration dependent decrease of the ESR signal intensity. The effect of Fe^{3+} was blocked with desferrioxamine, and the effect of Fe^{2+} was blocked with ferrozine in the system containing arginine and MG.

3.3. MG reduces Fe^{3+}

Reduction of Fe^{3+} by MG was investigated with a spectrophotometric method using ferrozine.

The formation of the Fe^{2+}–ferrozine complex was detected at 561 nm, in a Hitachi 2001 UV/visible double beam spectrophotometer, with and without desferrioxamine.

MG-induced Fe^{3+} reduction was prevented by desferrioxamine. Reduction of iron couldn't be detected in the system which failed MG.

3.4. Spectrophotometric analysis of the product of the arginine + MG system

The photometric spectra of the system arginine + MG, containing Fe^{3+} or Fe^{2+} (10 mM) were measured in a Hitachi 2001 UV/visible double beam spectrophotometer at visible wavelength (490 nm).

Both Fe^{3+} and Fe^{2+} caused an increase in a peak absorbing at 490 nm. Fe^{2+} catalyzed the colour development in the spectrophotometric examinations (at 490 nm) more effectively than Fe^{3+}.

3.5. The effect of smoke on the bradykinin-induced calcium increase

Endothelial cells, isolated from freshly prepared pig aortas were incubated with different dilutions and for different incubation time with tobacco smoke from cigarette passed through Krebs buffer (SB). Cells were also incubated with formaldehyde (ingredient of smoke) for 30 minutes with different concentrations (1-80 μM). The effect of GSH was examined in a co-incubation of 25% SB or 80 μM formaldehyde, after preincubation of different concentrations GSH.

Fura-2-AM was used for the measurement of $[Ca^{2+}]_i$. Calcium concentration was measured on cover glasses with confluent cell cultures, placed into quartz cuvettes, incubated with 250 nM bradykinin. Fluorescence measurement was performed at 37°C, using a Hitachi F-4500 fluorescent spectrophotometer. The excitation wavelengths were 340 and 380 nm; the emission wavelength was 510 nm.

A fluorescent method using *O*-phthaldialdehyde (OPA) was applied for the determination of GSH and protein thiol levels. After the treatment of the cells with formaldehyde (80 μM) or SB (50%) the fluorescence intensity was measured at 340 nm excitation and 425 nm emission wavelength. The difference of initial (without OPA) and final (with OPA) readings was compared with those in the corresponding GSH standards (0.625 to 10 μM).

We have measured protein-thiol concentration of the endothelial cells. Fluorescence was measured and the results were compared to the GSH standards. Protein content in the redissolved pellets of perchloric homogenates was measured with the method of Lowry et al. The results of GSH measurements are expressed as μmol/g protein. Protein-thiol is given as μEqu GSH/g protein.

Statistical significance was verified with Student's unpaired *t* test performed with the SPSS program package.

SB inhibited bradykinin-induced calcium increase. Calcium increase was not influenced by 6.25% and 12.5% dilution of SB, but was significantly inhibited by 25% and 50% of SB ($p<0.001$). The inhibition of the bradykinin-induced calcium increase by 25% SB was time-dependent. Twenty-five percent dilution of SB was effective after 10 min of incubation ($p<0.001$). The inhibitory effect of 25% SB on the bradykinin-induced calcium increase could be prevented by 4 mM GSH ($p<0.01$) and partially prevented by 2 mM GSH ($p < 0.05$). High concentrations (10, 20, 40, 80 μM) of formaldehyde did inhibit the calcium increase in a concentration-dependent manner ($p<0.05$ in all cases). GSH prevented the effect of 80 μM

formaldehyde. 50% SB decreased both GSH and protein-thiol levels. Eighty micromolar formaldehyde was ineffective in these cases.

3.6. The effect of Ald on ERK 1/2 and JNK phosphorylation in vascular smooth muscle cells (VSMCs)

Aortic VSMCs were isolated from Sprague-Dawley (SD) rats. Cells were treated with Ald (10^{-7} M) for different time. For Western blot experiments after incubation the membrane with primary antibody (polyclonal anti-phospho-ERK 1/2 and polyclonal anti-phospho-JNK, 1:1000) we have incubated them with secondary antibody (goat, peroxidase-conjugated, anti-rat IgG antibody, 1:5000). The intensity of ERK 1/2 and JNK phosphorylation was determined with densitometry.

Four to 6 cell stimulation experiments were performed and quantified. Ald (10^{-7} M) induced ERK 1/2 and JNK phosphorylation in VSMCs in time dependent manner, with maximal intensity after 10 minutes.

GSH (2 mM), Tiron (10 μM), and AG 1478 (EGFR blocker, $3x10^{-7}$ M, 10^{-7} M, 10^{-8} M) preincubation suppressed the Ald induced ERK 1/2 and JNK phosphorylation.

3.7. Ald potentiates angiotensin II–induced ERK 1/2 and JNK phosphorylation

Materials and methods, used for the experiments were the same as in previous subsection, supplemented with p42/44 assay kit and with fluorescence confocal microscopy. Both angiotensin II and Ald were used in 10^{-7} M concentration. The kinase assay for ERK 1/2 (p42/44 kinase assay) was performed with a kit from New England BioLabs as described in the protocol of the kit. The samples were applied for Western blot. The primary antibody was anti-phospho-Elk-1 antibody (1:1000), the secondary antibody was peroxidase-conjugated anti-rabbit antibody (1:2000). For the confocal microscopy, the cells were cultured onto covered glasses. The cells, after treatment with angiotensin II and Ald and incubation with antibodies, were embedded. Measu Quantification was performed with histogram function in the MRC Laser Sharp software. Data are presented as the mean fluorescence intensity in the respective cell area.

Angiotensin II-induced ERK 1/2 and JNK phosphorylation in VSMCs with a maximal intensity after 2 minutes. After Ald treatment the maximal intensity of ERK 1/2 and JNK phosphorylation was observed at 10 minutes. The combination of angiotensin II and Ald

resulted in a stronger ERK 1/2 phosphorylation at 2 minutes than with angiotensin II or Ald alone. The JNK phosphorylation was faster (after 1 minute) and stronger than with angiotensin II or Ald alone. We have confirmed the potentiating effect of Ald on angiotensin II-induced ERK 1/2 phosphorylation with confocal microscopy and p42/44 kinase assay too. We have investigated the phosphorylation of Elk-1, the substrate of ERK 1/2, with p42/44 kinase assay. Scavengers (GSH, Tiron) were able to suppress the phosphorylation of MAPKs after incubation with angiotensin II and Ald, alone or in the combination. The phosphorylation induced with combination were prevented with preincubation of AG 1478.

3.8. Mineralocorticoid receptor (MR) - blockers reduced the angiotensin II-induced ERK 1/2 phosphorylation and the end-organ damage in double transgenic rats for human renin and human angiotensinogen gene (dTGRs)

VSMCs were stimulated with angiotensin II (10^{-7} M) after preincubation with spironolactone (Spi, 30 min, 10^{-6} M). After stimulation, the samples were applied to Western blot analysis, p42/44 kinase assay and confocal microscopy experiment.

Intracellular free radical production was measured in rat VSMCs after incubation with DCF with fluorescence method. Cells were preincubated with Spi (10^{-7} M for 30 minutes) or DMSO (10^{-7} M for 30 minutes, as a control). Measurements were carried out with Zeiss fluorescence microscopy. The excitation wavelength was 488 nm, and the emission wavelength was 513 nm. VSMCs were analysed for MR expression with TaqMan polymerase chain reaction.

Experiments were conducted in 4-week-old male dTGR (n=28) and nontransgenic, age-matched SD (n=7) rats after due approval. For the Ald blockade dTGR group (n=14) received Epl for 3 weeks (100 mg, kg^{-1}, d^{-1} in the diet). Systolic blood pressure was measured by tail-cuff under light ether anaesthesia. Urine samples were collected over 24 hours. Urinary albumin was measured by ELISA. Echocardiography with M-mode and pulse-wave Doppler was performed with a commercially available system equipped with a 7-MHz phased-array transducer. Three measurements per heart were averaged. Rats were killed at the age of 7 weeks.

For the immunohistochemistry 6 μm cryosections were stained with primary monoclonal antibody (collagen type IV or phospho-ERK 1/2). The secondary antibody was anti-mouse IgG antibody. At the microscopic evaluation observers were unaware of the experimental

design and antibodies used. 15 area were examined with computerised cell-counting software (K 300 3.0, Zeiss) from each slides.

Spi decreased angiotensin II–induced ERK 1/2 phosphorylation at 10 minutes. We couldn't find the same reduction in angiotensin II-induced ERK 1/2 phosphorylation after 2 minutes (at the time point where the effect of angiotensin II is maximal). The results were confirmed with confocal microscopy to. In in vitro model Spi reduced the angiotensin II induced free radical production significantly after 5 minutes.

Systolic blood pressure was higher in non Epl-treated dTGR (n=14) compared to the Epl-treated dTGRs (n=14) and to the control, SD rats (n=7, $p<0.05$ in all cases). Urinary albumin excretion was markedly higher in dTGR than in SD rats (p=0,001). Epl treatment markedly reduced albuminuria in dTGRs (p<0,001). Epl-treatment reduced collagen IV matrix deposition in the kidney and heart. Untreated dTGR show ERK1/2 phosphorylation in the media of renal dTGR vessels, compared to the SD rat arteries, which increased phosphorylation was reduced by Epl. Epl also reduced cardiac hypertrophy index and improved left ventricular diastolic function (normalized E/A ratio) compared with untreated dTGR.

3.9. Effect of nutrition on the level of serum and urinary glycation end products

The effect of heat treated foods were investigated on the serum AGE level, urinary albumin excretion, creatinin clearance and blood pressure in a prospective, randomized, cross-over study, in 24 healthy volunteers. The serum and urine CML concentrations werw measured. The experiment was carried out with the permission of the Ethic Committee of the University of Pécs. Before the entry to the study, volunteers were informed from the study details and they had written informed consent.

In the first week of study every participant has had the same food as before. They should have a diet, containing small amount protein and keep a diary. To have a correct diet, they have a consultancy with dietician. After first week the volunteers were random by divided into two groups. The group „A" have had a high protein containing, heat-treated (cooked, boiled) diet on second week. The group „B" have had a high protein, raw diet. The third week was the wash out period for both groups, with the habitual food intake, and with recommendation to reduce the protein intake. On the fourth week each group have changed the diet compared to the second week. The group A get the raw, and group B get the heat-treated, high protein

containing diet. The protein content of the diet taken in the first and third week was calculated on the basis of diary. In the second and fourth week of the study the volunteers have the diet constructed by our dietician and prepared on the clinic. The protein content of the raw and heat treated diet was 3 g/kg/day (adjusted with ultrafiltrated protein concentration), which protein content is almost four times higher then the recommendation of the WHO. The CML content of the diet were calculated by the co-workers of the Institution of Nutrition on the Kiel University (Germany), based on their measurements. The heat treated diet have near to 60 mg/day CML intake, in contrast to the raw diet, practically without CML.

We have used 24 hour creatinine clearance to determine the glomerular filtration rate. The urinary albumin excretion was measured with immunoturbidimetry. The blood pressure was measured with 24 hour ABPM. Competitive ELISA was used to determine the serum and urine CML concentration. To estimate the absolute CML concentration, N^{ε}-carboxymethyl-amino-caproic acid served as standard. Absorbance was read in the microtiter ELISA plate reader (Multiscan Ascent, Labsystems) at 405 nm (reference: 603 nm) wavelength.

We have put together the results from the same dietary period of two groups. Data are expressed as mean ± standard error. Statistical significance was verified with Student's unpaired *t* test.

There was no significant difference in blood pressure (systolic, diastolic per day or per night, 24 hour mean blood pressure, diurnal index, hypertension impact and hypertension time index), in blood gases (pCO_2, pH, base excess, actual and standard bicarbonate), 24 hour creatinine clearance, C reactive protein, serum albumin, serum cholesterol and plasma fibrinogen level. The level of the serum CML was increased with CML rich, high protein containing diet. The serum CML was practically unchanged after CML poor, high protein containing diet. The urine CML excretion was significantly higher after CML rich protein load ($p < 0{,}0001$) compared to the CML poor diet. The urinary albumin excretion was increased after CML rich diet, but statistically remains not significant.

4. DISCUSSION AND CONCLUSIONS

4.1. Modification of L-arginine by methylglyoxal is catalyzed by iron redox cycling

Enhanced free radical- and/or aldehyde formation generate oxidative stress and/or carbonyl stress, inducing pathophysiologycal processes. The serum level of MG is higher in the diabetic patients then in healthy population. MG is produced by MG-synthase, cytochrom P450 IIEI and partially by non enzymatic pathway. Reaction of MG with arginine damages protein function through reaction with their amino groups. The reaction between MG and arginine induces imidazolone and or argpyrimidine formation.

Iron as a transitional metal potentiates free radical formation. The iron binding capacity of the serum of diabetic patients is reduced, in their storages the iron level is higher then in healthy peoples. Iron and MG induced calcium accumulation was higher in RBC model. The ESR signal intensity was enhanced with Fe^{3+} and reduced with Fe^{2+} in the MG-arginine reaction. Desferrioxamine reduced the effect of Fe^{3+}. Previous results suggest that Fe^{3+} receives electron from MG becoming reduced to Fe^{2+}. Ferrous iron can be chelated by MG and this complex reacts with L-arginine. Arginine is important in the NO production. It is substrate of NOS, this way have a role in the regulation of vascular system.

4.2. Effect of MG and Fe^{2+} on the intracellular calcium and oxidative stress in RBCs

The MG-induced oxidative stress evoked a calcium accumulation in RBCs. This effect of MG is similar to the effect of Fe^{2+}. Oxidative stress, intracellular calcium accumulation, higher serum MG level and increased intracellular iron level are all present in diabetic patients. The processes mentioned above, leads to the reduced RBC membrane deformability in this way generating damage of microcirculation and appearance of complications. Scavengers and desferrioxamine are capable to reduce MG and iron induced oxidative stress and calcium accumulation. Both oxidative stress and elevation of the intracellular calcium evoked by MG was ameliorated by desferrioxamine suggesting an important role of the extracellular iron or extracellular surface-bound iron of RBCs in these reactions. Extracellular iron may be strongly involved in the effect of MG because desferrioxamine is transported into the cells very slowly. The effect of MG was abolished with GSH in RBCs. GSH can act in two different ways. In addition to the scavenger property, glyoxal pathway have also

important role in the action of GSH. MG reduced the level of low molecular weight thiol content in our experiments, suggesting the role of oxidative stress. The data listed above support the hypothesis that MG may react with membrane proteins and lipids on the extracellular surface of the RBCs, causing intracellular oxidative stress, probably through a transmembrane process.

4.3. Effect of cigarette smoke and formaldehyde on the bradykinin-induced calcium accumulation and on the redox state of cells

Cigarette smoke and formaldehyde inhibit the bradykinin-induced calcium accumulation in concentration dependent manner. The inhibitory effect of smoke on the eNOS-NO-cGMP signalling pathway was not caused by superoxide or by hydroxyl free radical or by lipid peroxidation. GSH and other thiol group–containing agents proved to be protective. Oxidative stress, generated by cigarette smoke, may inhibit the agonist-induced calcium increase indirectly, due to the oxidation of thiol groups and cell membrane depolarization.

GSH prevented the inhibition caused by formaldehyde on the bradykinin-induced calcium increase. Smoke induces a reduction of the NO content in the tissues. This effect of smoke can be explained by oxidative stress and by changes of intracellular calcium level.

4.4. Effect of Ald on the angiotensin II induced ERK 1/2- and JNK phosphorylation in smooth muscle cell.

Angiotensin II and Ald induced ERK 1/2- and JNK phosphorylation in time dependent manner in the smooth muscle cells. These effect of angiotensin II and Ald can be blocked with scavengers and EGFR-blocker. EGFR has important role in the angiotensin II and Ald mediated cellular response, due to the participation in the angiotensin II induced free radical production and ERK 1/2 phosphorylation. The phosphorylation of JNK is free radical dependent but EGFR independent process.

Ald induced ERK 1/2 phosphorylation is detectable after few minutes, in smooth muscle cells. Ald enhanced EGF signalling, resulting in potentiated ERK 1/2 phosphorylation and intracellular Ca^{2+}-level. Angiotensin II-induced ERK 1/2- and JNK phosphorylation was potentiated in VSMCs with Ald and the agonists were additive. Angiotensin II and Ald induced MAPK phosphorylation was blocked with GSH and tiron. MAPK phosphorylation potentiated with angiotensin II and Ald is EGFR dependent. Previous data suggest that in the

angiotensin II and Ald induced pathway between NADPH oxidase and EGFR phosphorylation is common. Kinase such as src – which regulates trafficking of the EGFR out of the caveolae - may well be an interconnection between the two signalling pathways. Scavengers confirmed the important role of reactive oxygen specie in the ERK 1/2- and JNK phosphorylation.

Angiotensin II and Ald induces hypertrophy and fibrosis. Our experiments show, that the effect of angiotensin II and Ald in higher concentration is greater, they can potentiate harmful processes independently from classical renin-angiotensin-aldosterone pathway.

4.5. Effect of MR blocker on the angiotensin II induced ERK 1/2 phosphorylation in VSMCs and dTGR model

MR blocker Epl, used in dTGR experiments reduced urinary albumin excretion, systolic blood pressure, cardiac hypertrophy, collagen IV matrix deposition in heart, and improved left ventricular diastolic function. Epl reduced ERK 1/2 phosphorylation in the vessel wall of dTGRs. Connection between high serum Ald level and cardiac fibrosis is well known. MR is responsible for the sodium uptake in the cells and for the fibrosis, as well. Spi reduced the cardiac hypertrophy and fibrosis independently of systolic blood pressure. This observation suggests that Ald can generate cardiac fibrosis, independently form other members of RAAS.

Incubation of VSMCs with Ald increased the angiotensin receptor expression leading to greater diacylglycerol formation and intracellular calcium level. Epl was able to avoid the fibrosis induced by Ald. Our results suggest an interaction between angiotensin II and MR. The AT_1 receptor induced NADPH oxidase activation has two phases. The firs, quick $O_2^{\bullet}$-generation and other second phase, extended $O_2^{\bullet}$-production. Ald does not influence the early generation of angiotensin II–induced ROS production. The relevance of Ald-induced ROS production has also been shown in animal models. In aortic segments of Ald-infused rats, enhanced NADPH oxidase activity and NF-κB activation were found in addition to higher blood pressure and increased the mesenteric artery media/lumen ratio. The NADPH oxidase activity was higher in dTGRs and the NF-κB and AP-1 activation was greater also compared to the control.

Spi preincubation – in VSMCs - blocked the angiotensin II induced ERK 1/2 phosphorylation and the long-lasting NADPH oxidase activity. These results suggest that we can block the effect of angiotensin II before the EGFR activation. Based on previous results, not only blockade of AT_1 receptor but also the use of Ald receptor antagonist is necessary for

the appropriate cardiovascular protection.

4.6. Effect of heat treated, protein rich diet on serum AGE level and albuminuria in healthy volunteers

Glycation of arginine on high temperature leads to pyrazol and pyrimidine production. In our experiment after consumption of protein rich, heat treated (CML rich) diet, the fasting serum CML level and the urinary CML excretion was significantly higher. In the case the foods prepared without heat treatment (CML poor), the urinary CML excretion was decreased. The CML intake with every day diet is less then in diet prepared only with heat treatment, but more then in diet consist only of raw foods. The CML content of foods prepared with traditional cooking is the highest in fat rich foods and the lowest in carbohydrate rich foods. The presence of AGEs was confirmed in most tissues with CML detection. The AGEs role in pathophysiology appeared with connection to the RAGEs, this way initiating many processes. AGEs have important role in the development diabetic vascular complications, can induce programmed cell death, MAPK phosphorylation and NF- κB activation.

Under CML rich diet the creatinin clearance remained unchanged, but the urinary albumin excretion was increased. The AGE poor protein rich diet doesn't have effect on albuminuria. It means that the AGE content is important ingredient of the foods which can provoke albuminuria. In patients with chronic kidney diseases the higher AGE content can bee very harmful because of reduced excretion of AGE products. The previous results forced us to revise the techniques (long heat treatment, to take more raw vegetables and fruits, to avoid consumption of foods rich in fat, etc.) used for the preparation of foods and to popularize it. The data above suggest that the application of AGE poor diet should be very useful in the patients with chronic kidney disease, or nephropathy.

5. THESES

I. MG potentiates the free radical production, reduces the concentration of the low molecular weight thiol-content molecules and evokes intracellular calcium accumulation in RBC-s. In the presence of MG ferric iron is reduced to ferro iron.

II. Cigarette smoke inhibits bradykinin-induced calcium accumulation in time- and concentration dependent manner. Formaldehyde – a component of cigarette smoke – inhibited bradykinin-induced calcium increase. GSH prevented the effect of cigarette smoke and formaldehyde. Cigarette smoke decreased both the GSH and the protein-thiol level. We couldn't observe the same effect in case of formaldehyde.

III. Ald induced, time-dependent ERK 1/2 and JNK phosphorylation is transcription independent in smooth muscle cells. Ald induced ERK 1/2 and JNK phosphorylation is free radical dependent process.

IV. Ald potentiates angiotensin II induced MAPK phosphorylation. The angiotensin II induced MAPK phosphorylation, potentiated by Ald is free radical dependent.

V. The effect of angiotensin II induced MAPK phosphorylation, potentiated by Ald, was confirmed with transcription factors too (NF-κB, AP-1).

VI. Spi reduced angiotensin II induced ERK 1/2 phosphorylation. Spi reduced free radical production evoked by angiotensin II.

VII. The MR-blocker Epl reduced cardiac and renal fibrosis, the albuminuria, improved diastolic function and reduced the cardiac hypertrophy index in dTGRs. Epl reduced the ERK 1/2 phosphorylation in the kidney of dTGRs.

VIII. The raw foods with high protein content have low CML level and their intake reduced the level of CML in the urine.

IX. Eating cooked foods, with high protein content, which means increased CML intake, increases the serum and urine CML level.

X. High protein, cooked, CML rich diet increased the urinary albumin excretion.

6. The publications of the author used for the theses

Publications:

1. István Wittmann, **István Mazák**, László Pótó, Zoltán Wagner, László Wagner, Tibor Vas, Tibor Kovács, József Belágyi, Judit Nagy: Role of iron in the interaction of red blood cells with methylglyoxal. Modification of L-arginine by methylglyoxal is catalyzed by iron redox cycling. Chem Biol Interact. 2001; 138: 171-87. (IF:1.706)

2. István Mazák, István Wittmann, László Wagner, Zoltán Wagner, Péter Degrell, Tibor Vas, Gergő A. Molnár, Judit Nagy: Cigarette smoke and its formaldehyde component inhibit bradykinin-induced calcium increase in pig aortic endothelial cells. Endothelium 2002; 9: 103-108. (IF: 1.512)

3. István Mazák, Anette Fiebeler, Dominik N. Müller, Joon-Keun Park, Erdenechimeg Shagdarsuren, Carsten Lindschau, Ralf Dechend, Christiane Viedt, Bernhard Pilz, Herman Haller, Friedrich C. Luft: Aldosterone potentiates angiotenzine II-induced signaling in Vascular smotth muscle cells. Circulation 2004; 109: 2792-2800. (IF: 12,563)

4. Mazák István, Wittmann István, Wagner Zoltán, Wagner László, Nagy Judit: A dohányfüst gátolja a bradikinin kiváltotta kálcuim-beáramlást az endothel sejtekben. Hypertonia és Nephrologia 4: 37-41, 2000.

Congress abstracts:

5. **István Mazák**, István Wittmann, László Wagner, Zoltán Wagner, Judit Nagy: Smoke inhibits bradykinin-induced calcium influx in endothelial cells. Nephrol Dial Transplant 1999; 14: A10.

6. **István Mazák**, István Wittmann, László Wagner, Zoltán Wagner, Judit Nagy: Smoke inhibits bradykinin-induced calcium influx in endothelial cells. Cigarette-smokind and kidney involvement, 1999, Milano

7. **István Mazák**, István Wittmann, Zoltán Wagner, László Wagner, Tibor Vas, Judit Nagy: Methylglyoxal evokes oxidative stress and calcium accumulation in red blood cells. Exp Clin Endocrinol Diabetes 108: A6, 2000

8. **István Mazák**, István Wittmann, Zoltán Wagner, László Wagner, Tibor Vas, Judit Nagy: Methylglyoxal produced in non-enzymatic glycation evokes oxidative stress and calcium accumulation in human red blood cells. Nephrol Dial Transplant 2000; 15: A103.

9. **István Mazák**, István Wittmann, László Pótó, Zoltán Wagner, Tibor Kovács, László Wagner, Tibor Vas, József Belágyi, Judit Nagy: Role of iron and methylglyoxal in diabetic nephropathy. In vitro study using red blood cells. Nephrol Dial Transplant 2001; 16: A74.

10. István Wittmann, Zoltán Wagner, **István Mazák**, László Pótó, Reinhard Schinzel, August Heidland, Judit Nagy: Foods rich in advanced glycation end products (AGEs) induce microalbuminuria in healthy persons. Nephrol Dial Transplant 2001; 16: A106.

11. Zoltán Wagner, István Wittmann, **István Mazák**, László Pótó, Veronika Faist, Reinhard Schinzel, August Heidland, Judit Nagy: Effects of dietary carboxymethyllysine (CML) intake on CML levels and albumin excretion rates in healthy subjects. Diabetologia 2001; 44 (Suppl.1): A318.

12. **István Mazák**, Anette Fiebeler, Dominik N. Müller, Joon-Keun Park, Erdenechimeg Shagdarsuren, Carsten Lindschau, Ralf Dechend, Bernhard Pilz, Hermann Haller, Friedrich C. Luft: Aldosterone potentiates angiotenzin II-induced MAP Kinase signaling in vascular smooth muscle cells. Nephrol Dial Transplant 2003; 18 (Suppl. 4): 12.

13. Anette Fiebeler, **István Mazák**, Domink N Müller, Carsten Lindschau, Herman Haller, Friedrich Luft: Aldosterone induced non-transcriptional signaling in vascular smooth muscle cells. 29th International Aldosterone Conference, Philadelphia, USA, 2003

14. **Mazák István**, Wittmann István, Wagner Zoltán, Pótó László, Faist Veronika, Schinzel Reinhard, Heidland August, Nagy Judit. Hőkezelt ételek fogyasztásának hatása a glikációs végtermékek szérumszintjére és vizeletürítésére, valamint az albuminuriára. Magyar Belorvosi Archivum (Suppl.2): 78, 2001

7. ACKNOWLEDGEMENT

I am thankful to the **Almighty** who have supported me in the every day life, throughout the love and instructions of my **parents**, and love of my brothers. Thanks for the time of my **grandparents** spent with me and for things what I have learned form them. I get pleasure in every day life from my **wife**, and from my children's, from **Inke**, from **János** and from **Csongor**. I am grateful to my wife for the possibility to work on my theses.

I'm very thankful for the help of **István Wittmann** supported me in the design and execution my experiments. He has much time big patience to me and has attention for the people living not only in the clinical research. I'm grateful to professor **Judit Nagy** how have given me the possibility to start this work and for her advices, detailed instructions ad for the patience extended to the minor details.

I'm very grateful to **Béla Kocsis**, for the patience which, help me to do first steps in the experimental field, to the guide and selfless help what I find every day.

Thanks for the essential help of **László Wagner**, **Zoltán Wagner**, and **Molnár Gergő** in this work.

I get a big help in the lab work from **Ilona Sámikné** and **Anikó Heitmanné.**

I am thankful to **Tamás Szelestei,** to **László Belágyi,** to **László Pótó,** to **Tibor Kovács,** to **Péter Degrell,** to **István Pintér**, to **Matild Schmelczer**, to **György Fábián**, to **Nóra Szigeti**, to **István Késői,** to **Tibor Vas** and to **Judit Sebők.**

Thanks for the help to **Ildikó Udvarácz**, **Tünde Véber**, **Ibolya Kiss**, **Miklósné Szabó, Zsuzsanna Rumsauer** and **Enikő Bodor,** and to the **PhD-students.**

I am thankful to **Friedrich C. Luf**t how gave me the possibility to do experiments under very good conditions in Germany, where I could collect some experience and can close very successfully my experiments done there. I'm also grateful to the help and advice for **Dominik Müller**. I am thankful for the help to **Anette Fiebeler** and to **Ralf Dechend**. I am thankful for the help in the lab work to **Jürgen Janke, Carsten Lindschau**, **Gabriela N'diaye**, **May-Britt Köhler**, **Petra Quass** and **Matilde Schmidt.** Thanks for the help of **Susanne Wissler** on the administrative field.

Thanks for all others, mentioned not above, who help me in this work.