CLINICAL AND EXPERIMENTAL INVESTIGATION OF CONTINUOUS GLUCOSE MONITORING, OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

Doctoral (Ph.D.) theses

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1. ABBREVIATIONS

Ahsg	alpha-2 Heremans Schmid glycoprotein (fetuin A)
BMI	body mass index
BSA	bovine serum albumin
cGMP	cyclic guanosin-5'-monophosphate
CGMS	Continuous Glucose Monitoring
CSB	ciagerette smoke buffer
eNOS	endotelial nitrogen monoxide synthase
fruct	fructosamine
GSH	reduced glutathione
HbA_{1c}	haemoglobin A_{1c}
H_2O_2	hydrogen peroxide
NO	nitrogen monoxide
NOS	nitrogén monoxide synthase
NUC	non-uremic calciphylaxis
PBS	phosphate buffer solution
ROS	reactive oxygen substrate
SDS PAGE	sodium-dodecil-sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SSA	sulfosalicylic acid

2. SUMMARY

Number of type 2 diabetic patients increases from day to day - it is a world-wide well known phenomenon. Thanks to the novel therapeutic strategies the main causes of death are cardiovascular complications in these patients. The damage of the cardiovasculature begins to develop in the prediabetic conditions and it remains progressive. Therefore nowadays one of the main challanges of diabetology is the prevention and reduction of the progression of cardiovascular complications. We investigated how the individual therapy based on continuous glucose monitoring can help to achieve better glycaemic control and to decrease the risk of later diabetic complications. It is well known that oxidative stress plays crucial rule in the pathogenesis of diabetes mellitus and the development of diabetic complications. In our further investigations we established a novel method to monitoring oxidative stress processes. Then we revealed a possible new, so far unknown mechanism of the development of endothelial dysfunction induced by cigarette smoke. Finally with our case report we would like to draw attention to an irreversible and progressive vascular complication-the calciphylaxis.

3. INTRODUCTION AND AIMS

3.1. The achievement of tight glycaemic control is our therapeutic goal in the management of diabetic patients. Tight glycaemic control results in the increase of the number of hypoglycaemic episodes and it is well known that hypoglycaemia has a number of adverse effects. Therefore the declaration of individual therapeutic goals and ranges of glucometabolic parameters is very important. The fluctuations of blood glucose level play a crucial role in the development of diabetic complications. So the detection of these fluctuations must be our aim. Home blood glucose measurements provide only a snapshot picture of the whole-day blood glucose profile. Continuous interstitial glucose monitoring using glucose sensors allows achievement of adequate glycaemic control in diabetic patients. Our aims were to summarize and review the observations obtained by this new method (Continuous Glucose Monitoring System, CGMS) and to compare its benefits with the home blood glucose measurements. We investigated the accounts of CGMS in the detection of blood glucose fluctuations and the reveal of hypoglycaemia unawareness so the achievement of better glycaemic control.

3.2. Reactive oxygen species (ROS) have captured the interest of many researchers since they are thought to be associated with a lot of different physiological and pathological conditions. Under diabetic conditions the crucial role of oxidative stress in the development of hyperglycaemia induced complications is obvious. Endothelial dysfunction plays central rule in the pathogenesis of diabetic micro-, and macrovascular complications. Endothelial dysfunction is a consequence of hyperglycaemia induced oxidative stress processes and finally the decreased production of nitrogen monoxide (NO). Hydrogen peroxide (H_2O_2) is one of the ROS that are byproducts of several biochemical oxidative reactions. It is a relatively stable ROS, its amount is in micromolar range in human body. H_2O_2 is electroactive, accordingly voltammetric procedures have been also worked out and employed for its measurement. In our experiments a commercial, needle type amperometric glucose enzyme sensor manufactured for human patients was investigated. This sensor measures glucose through detecting H_2O_2 evolved in enzymatic reaction of glucose. Our aim was to inactivate the immobilized enzyme layer of the sensor producing a new sensor specific for H_2O_2-detection. The applicability, specificity, sensitivity, and the lower concentration limit of H_2O_2 detection were tested. Then we attempted to carry out in vitro and in vivo H_2O_2-measurements with this newly developed sensor in bovine serum albumin (BSA) and human plasma samples and in subcutan areas of anesthetized rats.

3.3. The investigation of the pathogenesis of cigarette smoke induced endothelial dysfunction has long history in the 2nd Department of Medicine and Nephrological Center in Pécs. Cigarette smoke contains large amounts of free radicals, prooxidants, and aldehydes, which are toxic to endothelial cells. There is overwhelming evidence that cigarette smoke causes endothelial dysfunction by decreasing the production and bioavailability of endothelial NO resulted in the disturbance of the endothel dependent vasodilatation. The pathomechanism of this process was unidentified. Nowadays it is well known that cigarette smoke can disturb the integrity of L-arginine-eNOS-cGMP-NO pathway at different levels generating endothelial dysfunction. The endothelial NO production depends among others on the post-translational modification of endothelial nitrogen monoxide synthase enzime (eNOS) which is regulated by –beside various cofactors- multisite phosphorylation. Regulation of eNOS activity involves highly coordinated phosphorylation and dephosphorylation processes. The role of the glutation redox system (GSH/GSSG) in the maintenance of cellular redox homeostasis is well known.

Our aims were to investigate the acute effects of cigarette smoke on eNOS phosphorylation in endothelial cell culture in vitro. We tried to reveal the acute effects of cigarette smoke extract on the phosphorylation of eNOS enzime at the activatory and inhibitory sites and finally to detect effects of the antioxidant reduced glutathione (GSH) on the eNOS enzime phosphorylations.

3.4. Acral gangraene can be generated by a potentially life–threatening clinical entity called calciphylaxis. It usually develops in patients with end–stage renal disease and/or hyperparathyreoidism, but more and more frequently in patients with normal kidney and parathyreoid function (so called non-uremic calciphylaxis, NUC). The exact pathogenetic factors are still unknown, hypofetuinaemia may play a role in its development. The key process of the syndrome is calcification of the tunica media in the small and medium–sized arterioles with concomitant intimal proliferation accompanied by ischaemia and necrosis.
We presented a unique case of a patient, who developed calciphylaxis after successful pancreas-kidney transplantation with normal renal and parathyroid function.

4. PATIENTS, MATERIALS AND METHODS

4.1. In the 2nd Departement of Medicine and Nephrological Center in Pécs continuous, 96 hour glucose monitoring was carried out in diabetic patients treated with insulin in a clinical trial sponsored by Lilly Hungary Ltd. Investigation started in February, 2003. Measurements were achieved by CGMS constructed by MiniMed Medtronic. Self-controlled monitoring was carried out in 29 diabetic patients (together 58 measurements). We examined patients on intensive-conservative insulin treatment with poor glycaemic control and good compliance. Two patients had insulin pump during the measurements. Most of the patients were type 1 diabetic (n=25), rest of them were type 2 diabetic (n=4). The patients clinical parameters were the followings: (mean ± SEM): age: 38 ± 3.2 years; body weight: 71.4 ± 2.2 kg; body height: 170 ± 2 cm; body mass index (BMI): 24.5 ± 0.6 kg/m^2; systolic blood pressure: 123 ± 2 Hgmm; diastolic blood pressure: 74 ± 2 Hgmm; hemoglobin A_{1c} (HbA_{1c}): 8.44 ± 0.34 %; fructosamine (fruct): 388 ± 15 µmol/l; total insulin dose: 49 ± 3 IU/day; insulin dose corraleted to body weight: 0.70 ± 0.04 IU/kg/day. Control measurements were carried out 7.6 months later (mean follow-up). Comparing the results of these measurements we analised the effects of the therapeutical changes on carbohydrate metabolism.

4.2. The commercial, needle type amperometric glucose enzyme sensor of CGMS was used for the experiments. The needle shaped sensor is made for measuring interstitial glucose through detecting H_2O_2 evolved in enzymatic reaction of glucose. The sensor contains all the three electrodes (working-, counter-, and reference electrode) needed for the amperometric detection. There are no detailed information about its structure or preparation technology**.** The simple battery powered, single purpose electronic unit was replaced by advanced electrochemical workstation served for our voltammetric measurements. A special connector had to be prepared for interconnection of the apparatus and the sensor. In the experiments the immobilized enzyme layer of the sensor was inactivated by 5-sulphosalicylic acid-2-hidrate (SSA) dissolved in PBS buffer, and the applicability of this "inhibited" glucose sensor for detecting H_2O_2 was tested. Voltammetric measurements were carried out in intensively stirred buffered aqueous media, in bovine serum albumin (BSA) and human plasma samples as well as in subcutan areas of anesthetized Wistar rats. Three months old Wistar male rats, weighing 250-300 g - were used in our experiments. Part of the animals were treated with streptozotocin (70mg x kg-1) i.p., and animals having a blood glucose concentration of at least 13 mM were selected as diabetic ones. Urethane was administered for anesthesia in rats.

4.3. Primary cultures of mouse endothelial cells from endothelioma were applied for our experiments (LGC Promochem, Taddington, UK). Cigarette smoke buffer (CSB) was produced by smoking commercial cigarettes with filter (Camel; R.J. Reynolds Tobacco) by a tube-driven apparatus into Krebs buffer as previously described. CSB solution was diluted with Krebs buffer (5% to 50%) and immediately applied to endothelial cells after preparation (for 5 to 30 min) to reveal the dose- and time-dependent acute effects of CSB compared with endothelial cells applied with Krebs puffer only on 37 ºC as controls. Furthermore endothelial cells were pretreated with GSH as antioxidant. Phosphorylated eNOS levels induced by CSB treatment were determined with Western blot analysis. Phosphorylation of the activatory (P-Ser(1177)-eNOS) and inhibitory site (P-Thr(495)-eNOS) was investigated. The phosphorylated protein levels were corrigated to the total eNOS levels and controls as well.

5. RESULTS

5.1. Significant correlation was observed between means of the data of the continuous glucose monitoring (8.3 ± 1.8 mmol/l) and the means of glucose levels measured by fingerprick (8.7 ± 2.1 mmol/l; r=0.682, p<0.001) for the first time. At the very beginning the patients mean HbA_{1c} level was 8.44 ± 1.79 %, and the mean fruct level was 388 ± 79 μmol/l. Significant correlations were observed between the glucose levels detected by this two methods and the long term parameteres of carbohydrate metabolism, like HbA_{1c} and fruct *(p<0.05)*. The mean of hyperglycaemic episodes was 8 episodes/96 hours/patient, the mean of hypoglycaemic episodes was 7 episodes/96 hours/patient, from which 3 episodes/96 hours/patient was unawared hypoglycaemia. Symptomatic and asymptomatic hypoglycaemic episodes were also detectable in most of the patients. According to our results the mean hyperglycaemic time index (the period spent in hyperglycaemia) was 27.85%, and the mean hypoglycaemic time index (the period spent in hypoglycaemia) was 12.11% for the first time.
Individual modification of the therapy was carried out according to the first measurements of CGMS which resulted in the improvement of the metabolic status in 25 patients, with a mean decrease of HbA_{1c}: 9.5% (p=0.001), fruct: 6.7% (p=0.017), interstitial glucose levels measured by CGMS: 1.96% (p=0.324) and blood glucose levels detected by glucometer: 2.5% (p=0.573). Meanwhile average decline of insulin doses was 7.3% (p=0.742), the decrease of carbohydrate intake was 6.6% (p=0.191) and the patients body weight (+0.8%; p=0.296) and BMI (+1.2%; p=0.244) did not altered respectively. Finally the worsening of glycaemic control was observed in our 4 non-compliance patients.

5.2. Firstly, in order to see the glucose and H_2O_2 measuring property of the glucose sensor the same amounts of glucose and H_2O_2 solutions we added and the addition was repeated. Amperometric measurements represented current increased stepwise. The difference of the electrode sensitivity was considerable, the sensitivity for H_2O_2 was approximately 400 times higher than in case of glucose according to the calibration curves. Then we soaked the sensor in 5% solution of SSA for 30 minutes for achieving enzime inactivation. In our further experiments we found that there was no current increase adding glucose to the solution after inactivation, indicating the total inactivation of the sensor. Similarly the ascorbic acid addition has not changed the current, indicating that the enzyme inactivation has not altered the selectivity of the newly developed H_2O_2-sensor. At the same time, the enzyme inactivation step has not changed significantly the H_2O_2-measuring property of the amperometric micro

sensor. The lower concentration limit of H_2O_2 detection attainable with the micro sensor was approximately 5µmol/l. The accuracy of the in vitro H_2O_2-measuring property of the sensor was checked in „recovery" experiments. The known and measured concentration values did not differ confirming the accuracy of H_2O_2-measurements. In further experiments the function of the amperometric H_2O_2-sensor was checked in two different biological media with known antioxidant capacity. In one case PBS buffer, containing 35g/l bovine serum albumin (known free radical scavenger) was pipetted into the measurement cell and the same amounts of H_2O_2-solution were added. The current increased stepwise and achieved a steady value after each addition, but the increase of it was smaller than that of obtained in simple PBS buffer. Repeating the same experiment with human plasma solution different observation could be made. Peak shaped transients were obtained indicating the fast decomposition of the H_2O_2. Higher dose of H_2O_2 resulted in higher peak height, however in plasma sample the current answer to H_2O_2 adding was much smaller than in BSA. Finally the H_2O_2 electrodes were implanted in diabetic and in healthy anesthetized rats. The current was followed for shorter and longer periods. While the blood glucose level of the diabetic rats were more than double, no significant difference could be detected between the H_2O_2-levels of the two groups in the areas of implantation. The detected current was very low and declined constantly.

5.3. CSB increased the phosphorylation of eNOS in a concentration-, and time dependent manner both at the activatory (Ser1177) and inhibitory site (Thr495). Maximal phosphorylation response to CSB occured with 50% CSB after 20 minutes long treatment. Moreover protein level of phospho-Thr(495)-eNOS was higher than that of phospho-Ser(1177)-eNOS at every concentration and time, while protein level of eNOS remained unchanged. Preincubation with GSH before CSB treatment decreased phosphorylation of eNOS at Ser(1177) by 20% and at Thr(495) by 45% at the same time.

6. DISCUSSION

6.1. The measurements carried out by CGMS proved to be adequate to detect the fluctuations of the glucose level which are often undetectable with the conventional blood glucose measurements. Correlations, similar to the literature were found between interstitial glucose levels measured by CGMS, blood glucose levels and long term glycaemic parameters. The number of unawared hypoglycaemic episodes was unexpectedly high detected by CGMS. We justified the relevance of CGMS in the detection of hypoglycaemia unawareness and especially the unawared nocturnal hypoglycaemic episodes. 7.6 months after the alteration of the therapy based on the first CGMS measurements, amelioration of the blood glucose levels, HbA_{1c} and fruct levels were detected in most cases. These were achieved by a slight lowering of insulin doses and carbohydrate-intake while the body weight did not changed. We found that CGMS can help to achieve better glycaemic control without enhancing the number of hypoglycaemic episodes. CGMS contributes to the development of individual insulin therapy and the achievement of better glycaemic control in diabetic patients. Therapetic changes based on CGMS measurements may contribute to decrease the risk of later diabetic complications. But this new method is established to complete but not to replace conventional blood glucose measurements. CGMS is obviously well adaptable to everyday use and also well tolerable by the patients.

6.2. The H_2O_2 is one of the most stable ROS serving as a markert to detect ROS generation and oxidative stress in vivo. Continuous in vivo monitoring of its level could be beneficial in research as well as in clinical practice. We found that the difference of the electrode sensitivity for glucose and H_2O_2 is considerable, the sensitivity for H_2O_2 is approximately 400 times higher than in case of glucose. The inactivation of the glucose oxidase enzim achieved by SSA was certified to be complete and the enzyme inactivation has not spoiled the selectivity for H_2O_2. The enzyme inactivation step has not changed the H_2O_2-measuring property of the micro sensor as well. The accuracy and reliability of the newly developed sensor was confirmed by further amperometric H_2O_2-measurements. Lower concentration limit of H_2O_2-measurement is comparable with the estimated concentration of H_2O_2 found in human body. Measurements carried out in known free radical scavanger BSA proved lower sensitivity for H_2O_2, indicating the actual free radical scavenging capacity and the higher viscosity of BSA. Repeating the same experiment with human plasma solution, peak shaped transients were obtained indicating the fast decomposition of the H_2O_2 and confirming the

very potent antioxidant capacity of the human plasma. The current recorded by in vivo implanted electrodes had a slow, but steady decreasing tendency indicating a slow declining sensitivity of the sensor most likely resulted from fibrin layer deposition on the sensor surface. For glucose measurements the manual of the glucose sensor requires separate blood glucose measurements and correcting the calibration factor of the implanted glucose sensor providing the accuracy of the glucose measurements. Declining sensitivity of the H_2O_2 sensor could be responsible for the unsuccessful H_2O_2 measurements in vivo. In summary we justified that the CGMS sensor measures selectively, sensitively and accurately low concentrations of H_2O_2 in presence of glucose and other electroactive species after an enzime inactivation procedure in vitro. Further studies are planed to gather detailed information about in vivo applicability of the H_2O_2-sensor. It is believed, that after further alterations the sensor will be applicable for following local changes of H_2O_2 level in anesthetized experimental animals as well as in human patients.

6.3. Endothel dysfunction is known to play an important part in the pathogenesis of multiple vascular diseases, such as hypertension, atherosclerosis, diabetes mellitus and chronic kidney disease. Impaired endothelial function has numerous consequences including the reduced NO production and bioavaibility and consequently impaired endothelium-dependent vasodilation. It is well known for a long time that cigarette smoke induces endothelial dysfunction. Smoking depletes the vasodilatory function of both micro-, and macrovascular endothelium. It is evidenced that cigarette smoke induces endothelial dysfunction by decreasing the production and bioavailability of endothelial NO, the exact pathomechanism of this process was unidentified so far. We investigated the effects of acute cigarette smoke treatment on the post-translational modifications of eNOS enzime in mouse endotheial cells. We firstly justified, that CSB treatment induces a shift of eNOS enzime activity to an inhibitory state which consequently leads to decreased NO bioavailability. Cigarette smoke contains large amounts of free radicals, prooxidants, and aldehides, which are toxic to endothelial cells triggering oxidative damage. GSH/GSSG is one of the main cellular redox systems maintains an optimal intracellular redox environment for proper function of cellular macromolecules. GSH treatment may result in the alteration of eNOS phosphorylation and shift of eNOS activity by reversing the effects of CSB consequently improving eNOS activity and endothelial function. GSH is assumed to improve the function of eNOS by consuming the aldehide components of cigarette smoke by its sulfhydryl groups. It might be supposed that

the influence of CSB on eNOS phosphorylation is mediated by aldehide components, through the modification of key thiol groups of the enzime.
We justified that cigarette smoke induced acut alterations in the activity of eNOS regulated by complex phosphorylation and decreased bioavailability of NO and endothel dependent vasodilation. These factors play very important part in the pathogenesis and progression of cardiovascular diseases. The positive effects of GSH confirm the role of aldehides found in cigarette smoke in the pathogenesis of endothelial dysfunction.

6.4. The appearance of cutaneous acral gangraene was observed in a 50 year-old woman, half a year after successful kidney-pancreas transplantation. Formerly she had suffered from type 1 diabetes mellitus for 45 years. Her renal function and the blood glucose level were normal therefore she did not need further dialysis and insulin therapy after transplantation when the gangrene appeared. Serum calcium, phosphorus and parathormon levels were also in normal range, but the serum fetuin level was lower and the insulin sensitivity was higher than normal. These factors have played probably an important role in the pathogenesis of calciphylaxis, and may have caused the appearance and worsening of clinical symptoms. Infusion therapy as well as increased dose of steroid resulted in an improve in microcirculation. Authors would like to draw attention to that gangraene caused by calciphylaxis can develop in post-uremic, non-diabetic patients with normal renal and parathyroid function in clinical remission phase.

7. THESES

1/1 CGMS proved to be adequate to detect more accurately the fluctuations of the glucose level compared to the conventional blood glucose measurements and reveal the unawared hypoglycaemic episodes which are often undetectable.

1/2 CGMS helps to develop individual insulin therapy and to achieve better glycaemic control in diabetic patients.

1/3 We justified that the alteration of the therapy based on the results of CGMS monitoring can improve glycaemic control and help to decrease the risk of later diabetic complications.

2/1 The glucose sensor of CGMS after an enzime-inactivation procedure will be able to carry out accurate, sensitive and selective detection of the changes of H_2O_2-level in vitro.

2/2 The lower concentration limit of H_2O_2-measurement was about 5µmol/l.

2/3 H_2O_2-measurements carried out in BSA and human plasma samples confirmed the antioxidant capacity of these biological samples.

3/1 We demonstrated for the first time that acute treatment with cigarette smoke buffer resulted in an increase of phosphorylation of eNOS both at activatory and inhibitory sites in a concentration- and time dependent manner. The amount of phosphorylated proteins at inhibitory site was higher compared to the activatory site of the enzime at each concentration and time.

3/2 The effect of CSB resulted in a shift of eNOS enzime activity to an inhibitory state which consequently leads to decreased NO bioavailability.

3/3 We justified that GSH diminished the CSB-induced increase in eNOS phosphorylation at both the activatory and inhibitory sites. The decrease of phosphorylation of the inhibitory site was higher.

3/4 GSH treatment may result in the shift of eNOS phosphorylation and activity by reversing the effects of CSB consequently improving NO bioavailability and endothelial function.

4/1 To our knowledge our patient was the first whose calciphylaxis was diagnosed after pancreas-kidney transplantation in non-diabetic condition with normal renal function.

4/2 We would like to draw attention that despite of successful pancreas-kidney transplantation the damage of vasculature remains irreversible and progressive in post-uremic, non-diabetic patients in clinical remission phase.

4/3 In our case the combined pentoxifylline and pentosan polysulfate infusions led to an improvement of microcirculation so this therapy might be suggested for patients with calciphylaxis.

8. PUBLICATIONS OF THE AUTHOR

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