The role of oxidative stress, subclinical inflammation and protein O-GlcNAcylation in diabetes mellitus, ischemia-reperfusion and chronic kidney disease

Summary of Ph.D. theses

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Pécs, 2011

ABBREVIATIONS

ACC: acetyl-CoA carboxylase
ACE-I: angiotensin converting enzyme inhibitor
ACO: aconitase 2
ACR: albumin creatinine ratio
AMPK: AMP-activated protein kinase
ARB: angiotensin receptor blocker
ASA: acetylsalicylic acid
ATP: adenosine triphosphate
BMI: body mass index
CA-N: cardiovascular autonomic neuropathy
cGMP: cyclic guanosine monophosphate
CKD: chronic kidney disease
CRP: C-reactive protein
CSB: cigarette smoke buffer
cTnI: cardiac troponin I
DAPI: 4',6 diamidino-2-phenylindole
EDP: end-diastolic pressure
eNOS: endothelial nitric oxide synthase
EPO: erythropoietin
FAT: fatty acid translocase/transporter; CD36
GFAT: glutamine: fructose-6-phosphate amidotransferase
GFR: glomerular filtration rate
GP: glycogen phosphorylase
GSH: reduced glutathione
H_2O_2: hydrogen peroxide
HBP: hexosamine biosynthesis pathway
HIF: hypoxia-inducible transcription factor
HPLC: high performance liquid chromatography
IL: interleukine
I/R: ischemia-reperfusion
KHB: Krebs-Henseleit bicarbonate
LDH: lactate dehydrogenase
LVDP: left ventricular developed pressure
MALDI-TOF/MS: matrix-assisted laser desorption/ionization - time of flight/ Mass Spectrometry
NADP: nicotinamide adenine dinucleotide phosphate
NAG-thiazoline: 1,2 dideoxy-2`-methyl-α-D-glucopyranoso(2,1-d)-Δ2`-thiazoline
NMR: nuclear magnetic resonance
NO: nitric oxide
OGA: β-N-acetylglycosidase; O-GlcNAcase
O-GlcNAc: O-linked β-N-acetylglucosamine
OGT: O-GlcNAc transferase
PF: pentoxifylline
PI3-K: phosphatidylinositol 3-kinase
PKA: cAMP-dependent protein kinase
PKB: protein kinase B/Akt
PKC: protein kinase C
PPS: pentosan polysulfate
PUGNAc: O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate)
RAS: renin-angiotensin system
ROS: reactive oxygen species
RPP: rate pressure product
Ser/Thr: serine/threonine
T2DM: type 2 diabetes mellitus
TCA: tricarboxylic acid
TNF: tumor necrosis factor
UDP-GlcNAc: uridine diphospho-N-acetyl-glucosamine
UDP-HexNAc: uridine diphospho-N-acetyl-hexosamine
ZDF: zucker diabetic fatty

INTRODUCTION AND AIMS

1. Oxidative stress and endothelial dysfunction: *In vitro* experiments on the cigarette smoke induced alterations in endothelial cells.

Impaired endothelial function is the major hallmark of several macrovascular diseases associated with accelerated atherosclerosis (e.g. ischemic heart disease, hypertension, diabetes mellitus, and chronic kidney disease (CKD)). In addition, microvascular endothelial dysfunction is closely associated with and could contribute to insulin resistance and microalbuminuria.

Cigarette smoking is an important risk factor in the progression of both macro- and microangiopathy. Cigarette smoke induces oxidative stress via enhanced ROS (reactive oxygen species) leading to several damaging effects in the targeted vascular endothelium; the most important of which is the decreased endothelium-dependent vasodilation due to reduced nitric oxide (NO) availability, typically through decreased eNOS (endothelial nitric oxide synthase) activity. Regulation of eNOS activity is relatively complex; however, it is well-recognized that phosphorylation of eNOS at Ser(1177) increases, whereas at Thr(495) it inhibits eNOS enzyme activity and NO production. While we and others showed that cigarette smoke causes endothelial dysfunction by disturbing the integrity of eNOS - NO - cGMP/cyclic guanosine monophosphate/ pathway at different levels; the effects on eNOS posttranslational modifications and the role of responsible protein kinases are unknown.

Our objectives were to determine:

(i) the acute effects of cigarette smoke buffer (CSB) on eNOS phosphorylations and eNOS dimer formation;

(ii) whether reduced glutathione (GSH) prevented the CSB-induced changes to eNOS modifications;

(iii) the role of protein kinase pathways in mediating CSB-induced changes in eNOS phosphorylations using selective PKA (cAMP-dependent protein kinase), PI3-K (phosphatidylinositol 3-kinase)/PKB (protein kinase B), and PKC (protein kinase C) inhibitors;

(iv) whether the isoform specific PKCβII-inhibitor, ruboxistaurin exerted protective effects on eNOS phosphorylations in response to CSB;

2. Oxidative stress and cardiac dysfunction: *Ex vivo* experiments on the role of protein O-GlcNAcylation in mediating cardioprotection against ischemia-reperfusion injury in the isolated perfused rat heart.

Chronic activation of the hexosamine biosynthesis pathway (HBP) and resulting increases in O-GlcNAc (O-linked β-N-acetylglucosamine) have been perceived as detrimental in the pathogenesis of glucose toxicity and insulin resistance. Paradoxically, accumulating evidence shows that acute activation of these pathways affords protection against a wide range of injuries in the cardiovascular system.

The posttranslational modification of proteins by O-GlcNAc (O-GlcNAcylation) is increasingly recognized as a novel signal transduction mechanism regulating diverse cellular functions (e.g. nuclear transport, translation, transcription, cytoskeletal reorganization, proteasomal degradation, apoptosis). It involves the reversible addition of a single O-GlcNAc moiety to Ser/Thr residues of nuclear and cytosolic proteins and is regulated by two key enzymes: O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc, and β-N-acetylglucosaminidase (O-GlcNAcase, OGA), which catalyzes its removal. OGT activity is sensitive to the intracellular concentration of UDP-GlcNAc (uridine diphospho-N-acetyl-glucosamine), which is the end-product of the HBP. Flux through the HBP and synthesis of UDP-GlcNAc is regulated in large part via the metabolism of glucose by GFAT (L-glutamine-D-fructose 6-phosphate amidotransferase). Glucosamine, which enters cells via the glucose transporter system and is phosphorylated to glucosamine-6-phosphate by hexokinase (bypassing the rate-limiting enzyme GFAT) rapidly increases UDP-GlcNAc, and thus O-GlcNAc levels. Protein O-GlcNAcylation can be increased by inhibiting OGA with PUGNAc (O-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-N-phenylcarbamate) or NAG-thiazoline derivatives [e.g. NBt: 1,2 dideoxy-2`-propyl-α-D-glucopyranoso-(2,1-d)-Δ2`-thiazoline; NAe: 1,2 dideoxy-2`-ethylamino-α-D-glucopyranoso-(2,1-d)-Δ2`-thiazoline].

Several *ex vivo* and *in vivo* studies showed that glucosamine and PUGNAc by increasing O-GlcNAc protected the heart against ischemia-reperfusion (I/R) injury. However, the lack of specificity and other off-target effects may give rise to disparate physiological actions, thereby limiting their value and potential clinical utility. Recently, novel NAG-thiazolines have been developed that have higher selectivity for OGA. For example, NBt showed ~1500-fold greater specificity than PUGNAc, whereas NAe is more potent (~30-fold) than NBt. Although neither NBt nor NAe has been tested in I/R injury at whole organ level. Despite a growing body of reports showing overall changes

in cardiac O-GlcNAc in response to different pathological stresses, subcellular distribution of O-GlcNAc in the intact adult heart or how it is affected by ischemia and I/R is not known. There have been also scarce data on those intracellular O-GlcNAc-signaling targets during ischemia and/or reperfusion that may contribute to myocardial preservation.

We purposed:

(i) to verify that selective inhibition of OGA with NBt and NAe at the time of reperfusion attenuates I/R injury in the isolated perfused heart;

(ii) to determine the effects of ischemia, I/R, and OGA inhibition on cardiac O-GlcNAc distribution;

(iii) to determine whether OGA inhibition prevented the changes in myocardial structural integrity and Z-line proteins following ischemia and I/R;

(iv) to identify protein targets altered by O-GlcNAc in response to ischemia and I/R;

3. Investigations on the role of increased HBP flux and protein O-GlcNAcylation, micro-inflammatory state and oxidative stress as contributing factors to the pathogenesis of diabetes-related complications and chronic kidney disease.

3.1. *Ex vivo* experiments on the effects of increased HBP flux and protein O-GlcNAcylation on the regulation of cardiac metabolism.

Cardiovascular complications are the leading cause of excess premature morbidity and mortality in diabetic patients. An early consequence of diabetes and insulin resistance in the heart is maladaptive metabolism, namely the increased fatty acid oxidation and suppressed glucose metabolism, which contributes to the development of diabetic cardiomyopathy.

Diabetes is known to increase O-GlcNAc in several tissue types (e.g kidney, cardiac tissue). Sustained activation of the HBP and protein O-GlcNAcylation has been implicated in the diabetic vascular-endothelial dysfunctions associated with increased oxidative stress and inflammation. Increasing the HBP flux and O-GlcNAc by glucosamine has been shown to mimic some of the diabetes induced adverse changes on the heart; however, the effect on cardiac metabolism is unknown. In adipocytes, glucosamine led to increased palmitate oxidation via activation of AMPK (AMP-activated protein kinase) and ACC (acetyl-CoA carboxylase) in an O-GlcNAc-dependent manner.

Our goals were to determine whether:

(i) activation of the HBP and O-GlcNAcylation with glucosamine altered cardiac metabolism similar to that seen in the diabetic heart;

(ii) activation of AMPK, ACC or fatty acid transporter (FAT)/CD36 was responsible for the altered cardiac substrate utilization;

(iii) increased membrane-associated FAT/CD36 could be a direct result of O-GlcNAc modification;

3.2. *Clinical study* to investigate effectiveness of the anti-inflammatory pentoxifylline and pentosan polysulfate combination therapy on diabetic neuropathy and albuminuria in type 2 diabetic patients.

The mechanisms underlying diabetes-related vascular complications are multiple; however, particularly in type 2 diabetes (T2DM), the increased oxidative stress and chronic inflammation are important pathogenic factors. Increased inflammatory activity is closely related to the onset and progression of both cardiac autonomic dysfunction and urinary albumin excretion, the surrogate symptoms of diabetic neuropathy and nephropathy, respectively. Adverse effects of inflammation (increased levels of pro-inflammatory cytokines growth factors, adhesion molecules) have been linked to altered vasoregulatory responses, increased vascular permeability, leucocyte adhesion, and facilitated thrombus formation leading to microvascular dysfunction such at the level of vasa nervorum or the glomeruli. In diabetes, adverse hemorheological features (e.g. hypercoagulability, hyperviscosity) and red blood cell abnormalities (e.g. impaired deformability, reduced O_2-binding capacity, increased aggregation) could also complicate the microangiopathy.

In face of multiple pathogenic factors and co-morbidities in diabetic neuropathy and nephropathy, specific treatments are not feasible. A number of new promising drugs have recently gained interests that potentially could delay the progression of diabetic nephropathy, including pentoxifylline (PF) and pentosan polysulfate (PPS); although their potential benefits in diabetic neuropathy have not been examined. Both PF and PPS have been shown to possess significant pro-circulatory, anti-inflammatory, and anti-proteinuric effects, thus these drugs could be symptomatic and in part, causal approaches to attenuate microvascular complications.

We purposed to determine the beneficial effects of combined PF and PPS infusion therapy in patients with T2DM:

(i) on cardiovascular autonomic and peripheral sensory functions (as regard with neuropathy);

(ii) and urinary albumin excretion (as regard with nephropathy);

3.3. *Clinical study* to investigate erythropoietin resistance and the effects of the anti-inflammatory acetylsalicylic acid on anemia correction in type 2 diabetes mellitus and chronic kidney disease.

Anemia, a frequent complication of T2DM and CKD, has been mostly attributed to decreased EPO (erythropoietin) synthesis due to the loss of renal interstitial fibroblasts; however, unresponsiveness to EPO has been also advanced. Increased pro-inflammatory state and oxidative stress, both of which are characteristic features of T2DM and CKD, have been put forward to explain EPO resistance. It would be predicted that patients with diabetic nephropathy showing higher levels of pro-inflammatory markers and oxidative stress exhibit higher degree of EPO resistance.

While EPO replacement therapy has been widely used for improving the availability of EPO and anemia, the effects of anti-inflammatory agents on serum EPO levels and erythropoiesis are not known. ROS are known to mediate intracellular signaling and transcriptional events through redox-sensitive pathways; one such example is the regulation of renal EPO secretion via HIF-1 (hypoxia-inducible transcription factor) and hydroxyl free radicals as the second messengers. It is conceivable that increased ROS associated with T2DM and CKD may critically alter the oxygen sensing regulatory mechanisms leading to disturbed HIF-1 activation and suppressed EPO synthesis. If so, a potent anti-inflammatory and hydroxyl free radical scavenger, such as ASA (acetylsalicylic acid) may be advantageous to improve EPO production, thus anemia in patients with T2DM and CKD.

Our aims were to determine:

(i) the presence of EPO resistance in patients with T2DM and/or CKD;

(ii) whether ASA corrected low EPO levels and anemia in a subgroup of patients with T2DM and CKD;

SUBJECTS, MATERIALS AND METHODS

1) Endothelial cells (from mouse endothelioma cell line) were incubated with either Krebs-Henseleit bicarbonate (KHB) buffer (Control) or increasing CSB concentrations (5 - 50%) for 5 - 30 min to detect dose- and time-dependent changes in eNOS posttranslational modifications. CSB was prepared in a tube-driven apparatus where cigarettes (Camel®) were smoked by a constant vacuum (-5 cmH_2O) for 5 min, then smoke was driven into 5 mL KHB. Cells were pre-incubated with GSH (5 mM; 15 min) in the antioxidant experiments. To determine the role of protein kinase pathways, the following protein kinase inhibitors were used: 1) selective PKA inhibitor (H-89; 10 µM); 2) selective PI3-K/PKB inhibitor (LY-294002; 100 µM); 3) selective PKC inhibitor (Ro-318425; 1 µM); and 4) isoform specific PKCβII-inhibitor (Ruboxistaurin; 30 nM). In immunoblot analyses, phospho-Ser(1177) and phospho-Thr(495) eNOS, total eNOS, phospho-Ser(473) and total Akt, as well as dimer eNOS levels were determined followed by densitometric and statistical analyses (one-way ANOVA-Dunnett`s posthoc test, unpaired t-test, and Jonckheere-Terpstra test; SPSS 10.0, SPSS Inc.)

Animal experiments below were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and conformed to the Guide for the Care and Usage of Laboratory Animals (NIH Publication No. 85-23, 1996).

2) After ketamine anesthesia, isolated hearts from non-fasted, male Sprague-Dawley rats were perfused retrogradely in a modified Langendorff model under standard conditions using KHB buffer containing glucose as the sole substrate. Coronary flow was adjusted to maintain a constant perfusion pressure of 75 mmHg. Cardiac functions were monitored via a fluid-filled balloon inserted into the left ventricle, connected to a pressure transducer and were registered by a heart performance analyzer. EDP (end-diastolic pressure) was set to 5 mmHg by adjusting the balloon volume. Cardiac performance was determined by left ventricular developed pressure (LVDP = maximal systolic pressure - EDP), maximum/ minimum rate of LVDP (± dP/dt), and rate-pressure product (RPP = heart rate X LVDP). After 30 min equilibration, hearts were randomly assigned to either a time-control, normoxia group (Norm) or four I/R groups subjected to 20 min no-flow ischemia followed by 60 min reperfusion. At the time of 60 min-reperfusion I/R hearts were either 1) untreated (Control) or treated with 2) 50 µM NBt (NBt50), 3) 100 µM NBt (NBt100), 4) 50 µM NAe (NAe). Pressure signals were analyzed for ventricular tachycardia (VT, four or more consecutive ectopic beats) and fibrillation (VF, fast mechanical activity with

barely discernible beat and minimal LVDP) during reperfusion. Cardiac troponin I (cTnI) release was determined in pooled coronary effluents collected at 15, 30, 45 and 60 min of reperfusion using ELISA kit. ATP and UDP-HexNAc levels were analyzed by HPLC in perchloric acid extracted frozen heart tissue (UV detection, 262 nm).

Tissue distribution of O-GlcNAc, desmin and vinculin were determined by indirect immunofluorescence using epifluorescence microscope. Additional group of hearts was subjected to 20 min ischemia without reperfusion to compare ischemia and I/R-induced changes. Sections were incubated with primary antibodies for O-GlcNAc (CTD110.6), desmin, and vinculin then proteins were visualized with Alexa Fluor conjugated secondary antibodies with DAPI (4',6 diamidino-2-phenylindole) to identify nuclei. For immunoblot analyses, frozen pulverized heart tissue was homogenized to obtain whole cardiac lysates, and nuclear, cytosolic, membrane, or mitochondrial fractions. Immunoblots were probed and visualized for O-GlcNAc (CTD110.6), OGT, phospho-Tyr(822) and total vinculin, desmin, aconitase 2 (ACO), glycogen phosphorylase (GP) followed by densitometric analyses.

O-GlcNAc-associated proteins for identification were determined by Western blot (CTD 110.6) then corresponding protein bands of Coomassie-stained gels were excised, trypsin digested and processed for MALDI-TOF Mass Spectrometry. Peptide mass spectra were analyzed (Voyager Explorer) and searched against Swiss-Prot database (Mascot software). Additional groups of hearts were: 1) time-matched, normoxic (Normoxia); 2) ischemic (Ischemia, 10 min); and 3) reperfused after 10 min ischemia (Reperfusion, 60 min). O-GlcNAc modification of vinculin, GP, and ACO as well as their association with OGT were determined by immunoprecipitation. ACO activity was measured spectrophotometrically (340 nm) by the conversion of citrate to α-ketoglutarate coupled to the reduction of NADP.

Statistical analyses included two-way and one-way ANOVA (Bonferroni's posthoc test), Pearson's correlations, X^2-test, and unpaired t-test (Prism 4.0, GraphPad Inc.).

3.1) Isolated hearts from non-fasted, Sprague Dawley male rats were perfused as described in *Section 2*. Perfusion KHB buffer contained (in mM) glucose 5.0, lactate 1.0, pyruvate 0.1, palmitate 0.32, glutamine 0.5, and 50 μU/mL insulin. Hearts were randomly assigned to one of six groups for a 60 min normoxic perfusion with the following glucosamine concentrations: 1) 0 mM (n=8); 2) 0.05 mM (n=5); 3) 0.1 mM (n=8); 4) 1.0 mM (n=4); 5) 5.0 mM (n=8); and 6) 10.0 mM (n=7). Hearts were perfused with [U-^{13}C]-palmitate, [3-^{13}C]-lactate, and [2-^{13}C]-pyruvate for the final 40 min of

protocol. ^{13}C-NMR isotopomer analyses of acid extracted hearts were performed to determine the relative contribution of energy substrates to tricarboxylic acid (TCA) cycle. ^{1}H-NMR spectroscopy was used to determine the lactate efflux and uptake rates. ATP and UDP-HexNAc levels were determined by HPLC method. Pulverized frozen hearts were homogenized to obtain whole tissue or membrane fraction lysates. Immunoblots were stained and visualized for O-GlcNAc (CTD110.6), phospho-Ser(79) and total ACC, phospho-Thr(172) AMPK-α and total AMPK-α, and membrane-associated FAT/CD36 levels. After FAT/CD36 immunoprecipitation, O-GlcNAc (CTD 110.6), OGT, and FAT/CD36 levels were determined. Statistical analyses were one-way ANOVA (Dunnett`s posthoc test), and unpaired t-test (Prism 4.0, GraphPad Inc).

<u>Clinical studies below were approved by the Ethical Committee of Medical Faculty, University of Pécs, and all patients gave written informed consent.</u>

3.2) This placebo-controlled study included two groups of in-patients with T2DM (≥ 1 year duration) and the symptoms of distal peripheral sensory neuropathy: 1) treated patients (<u>Verum</u>; n=77), who daily received combined PF (100 mg, Trental®) and PPS (100 mg, SP54®) infusions for 5 days; and 2) untreated, control subjects (<u>Placebo</u>; n=12) were given only saline (500 mL) infusions in the same regimen. Controls were matched for age, gender, T2DM duration, BMI, hemoglobin A_{1c}, and renal function. ACE-I and/or ARB, statin, or anti-platelet therapy did not differ between the groups. Exclusion criteria were as follows: neuropathies with other etiology, advanced liver failure, CKD (serum creatinine >200 µmol/L), heart failure, arrhythmia, hypertension (>160 mmHg), severe diabetic retinopathy, bleeding, disability, incompliance. Examinations were performed at hospitalization on the 1st (before) and 5th day (after therapy). CA-N (cardiovascular autonomic neuropathy) was assessed using five standardized reflexes described by Ewing; including the assessments of heart rate responses [deep breath test (expiration/inspiration ratio), Valsalva maneuver (Valsalva ratio), and to standing up (30/15 ratio)] and blood pressure responses (i.e. postural systolic fall, and diastolic rise to handgrip). The individual and summarized results (i.e. autonomic score) of tests were evaluated in age-matched categories using Innobase 3.1 software attached to ECG. Vibration perception of lower limbs was assessed with 128 Hz Riedel-Seiffer calibrated tuning fork. Albuminuria was measured by nephelometry from single urine samples and albumin/creatinine ratio (ACR) was calculated. Statistical analyses included non-parametric (Wilcoxon, Mann–Whitney U),

parametric tests (unpaired- and paired t-test), X^2-test, and Spearman's correlation (SPSS 10.0, SPSS Inc).

3.3) We examined EPO resistance in a cross-section study by comparing four groups of subjects with comparable serum EPO levels: 1) T2DM patients with normal glomerular function (defined as serum creatinine: 60–120 µmol/L and GFR: 90–120 mL/min) [DM; n=15]; 2) T2DM patients with nephropathy (DM+CKD; n=15); 3) non-diabetic patients with CKD (CKD; n=15); and 4) healthy individuals matched for age and gender (CONTR; n=10). There were no differences in glycemic control between the DM and DM+CKD groups measured by hemoglobin A_{1c}, fructosamine, and plasma glucose. Renal function measured by serum creatinine and GFR did not differ between the CONTR and DM groups, or the DM+CKD and CKD groups. In the CKD group etiologies of kidney diseases were as follows: hypertensive nephropathy (n=9), chronic pyelonephritis (n=3), analgesic nephropathy (n=1), chronic glomerulonephritis (n=1), and polycystic kidney disease (n=1). Anemic patients with malignancy, autoimmune disease, or deficiencies were excluded. In the subsequent intervention study, a subgroup of anemic patients with low serum EPO levels was included (DM: n=3; DM+CKD: n=4; CKD: n=3) and received per os 1 gram ASA (Aspirin®). Exclusion criteria were smoking, hyperacidity, gastric/duodenal ulcer, gastrointestinal bleeding, malignancy, chronic pulmonary disease. Fasting blood samples were collected at baseline and after ASA administration at the 4th, 8th, 24th, and 48th hour. Serum EPO, reticulocyte count and ratio, red blood cell count, hematocrit, hemoglobin, and lactate dehydrogenase (LDH) were measured by standard laboratory methods. Statistical analyses involved one-way ANOVA (Bonferroni`s posthoc test), unpaired- and paired t-test, $X^2$-test, and Pearson`s correlation (SPSS 10.0, SPSS Inc.).

RESULTS

1. (i) CSB augmented both phosho-Ser(1177) and phospho-Thr(495) eNOS levels in a concentration- ($p<0.05$) and time-dependent manner ($p<0.05$) compared to untreated controls with a maximal response at 50% CSB after 30 min. Phospho-Thr(495) eNOS levels were significantly higher at all concentration and time points (*$p<0.05$ vs. phospho-Ser(1177)*). CSB-induced disruption of homodimeric eNOS was also evident in a concentration- ($p<0.05$) and time-dependent manner ($p<0.05$) as indicated by decreased dimer/monomer eNOS ratios.

(ii) GSH attenuated the CSB-induced increases in phospho-Ser(1177) (~20%) and phospho-Thr(495) eNOS (~45%) levels compared to GSH-untreated cells (*p<0.05*). Reduction in eNOS phosphorylations was more pronounced at the Thr(495) residue (*p<0.05 vs. Ser(1177)*). GSH prevented the disruption of eNOS dimers (*p<0.05*) that was more apparent at the peak CSB effect (50%, 20 min), at which GSH reversed the dissociation of homodimeric eNOS and the ratio of dimer/monomer eNOS remained comparable that seen in CSB-untreated controls (*NS*).

(iii) CSB inhibited the PKB/Akt phosphorylation at Ser(473) in a concentration- (*p<0.05*) and time-dependent manner (*p<0.05*). After CSB, selective PI3-K/Akt inhibitor (LY-294002) further enhanced phospho-Ser(1177) eNOS compared to untreated controls (*p<0.05*), and it had no impact on phospho-Thr(495) eNOS (*NS*). Using selective PKA inhibitor (H-89), we did not find changes in eNOS phosphorylations at Thr(495) or Ser(1177) sites (*NS*). Selective PKC inhibitor (Ro-318425) significantly reduced phospho-Thr(495) eNOS at all CSB concentrations (10%, 20%, 50%; *p<0.05*), and it also increased phospho-Ser(1177) eNOS only at 50% CSB (*p<0.05*).

(iv) The isoform specific PKCβII-inhibitor, ruboxistaurin significantly suppressed phospho-Thr(495) eNOS levels regardless of CSB concentrations (*p<0.05*), and it increased phospho-Ser(1177) eNOS levels after 50% CSB treatment (*p<0.05*). Ruboxistaurin increased the ratio of phospho-Ser(1177)/phospho-Thr(495) eNOS in a concentration-dependent manner (*10 and 20% CSB: p<0.05; 50% CSB: p<0.001*).

2. (i) I/R hearts showed markedly declined cardiac functions (LVDP, ± dP/dt and RPP), UDP-HexNAc and ATP levels, and increased cTnI release (*p<0.05 vs. Norm*), and this was accompanied by a ~50% loss in overall protein O-GlcNAcylation (*p<0.05 vs. Norm*). Baseline cardiac functions did not differ between untreated, I/R hearts (Control) and those treated with NBt50, NBt100 and NAe during reperfusion. Recoveries of RPP, LVDP, and max dP/dt during reperfusion were higher in NBt100 and NAe groups (*p<0.05 vs. Control*), and were apparent as early as 5 min and maintained throughout the reperfusion in the NAe group. At the end of reperfusion RPP, LVDP and ± dP/dt were all higher in NBt100 and NAe groups (*p<0.05 vs.Control*) and were significantly higher in the NAe group compared to both NBt50 and NBt100 groups (*p<0.05*). Both NBt100 and NAe markedly reduced cTnI release (*p<0.05 vs. Control*), and NAe-treated hearts showed lower cTnI compared to both NBt50 and NBt100 groups (*p<0.05*). During reperfusion incidence of VT and/or VF was 86% in the untreated Control group, 14% in the NBt50 group, and was absent in both NBt100 and

NAe groups ($p<0.05$). There were no differences in UDP-HexNAc and ATP levels between any of the I/R groups regardless of treatments. NAG-thiazolines largely increased overall O-GlcNAc in all treatment (NBt50, NBt100 and NAe) groups ($p<0.001$ *vs. Control*). O-GlcNAc was higher in the NBt100 group compared to NBt50 group ($p<0.05$), and was higher in the NAe group compared to both NBt50 and NBt100 groups ($p<0.05$). There were significant correlations between O-GlcNAc and RPP ($R^2 = 0.82$; $p<0.05$), max dP/dt ($R^2 = 0.77$; $p<0.05$), and cTnI ($R^2 = 0.65$; $p<0.05$), and between RPP and cTnI ($R^2 = 0.83$; $p<0.05$).

(ii) In normoxic hearts, O-GlcNAc immunofluorescence was particularly intense within the nuclei of cardiomyocytes and exhibited a clear cross-striated pattern in the cytoplasm. Ischemia alone had relatively little effect on overall O-GlcNAc staining and the striated pattern remained unchanged; however, ischemia led to prominent changes in nuclear O-GlcNAc distribution (i.e. appearance of punctate and perinuclear staining of otherwise O-GlcNAc negative nuclei). Following I/R, there was a marked loss of O-GlcNAc fluorescence in both cytoplasmic and nuclear compartments, together with a loss of striated pattern, while the changes in nuclear O-GlcNAc staining as seen with ischemia alone were also evident. NBt50 treatment augmented O-GlcNAc fluorescence and preserved the cross-striated O-GlcNAc pattern in the cytosol compared to untreated I/R hearts. However, nuclear O-GlcNAc staining remained low, and punctate and perinuclear O-GlcNAc staining were still present. By immunoblot analyses, I/R reduced O-GlcNAc by ~50% in both nuclear and cytosolic fractions ($p<0.05$ *vs. Norm*). NBt50 increased the cytosolic ($p<0.05$ *vs. Control*) but not the nuclear O-GlcNAc levels (*NS*). NBt100 and NAe increased both nuclear and cytosolic O-GlcNAc ($p<0.05$ *vs. Control*), although increases in nuclear O-GlcNAc were blunted ($p<0.05$ *vs. Cytosolic*). I/R markedly reduced OGT in both nuclear and cytoplasmic fractions ($p<0.05$ *vs. Norm*). NBt50, NBt100 and NAe attenuated the loss of OGT in the cytosolic fraction ($p<0.05$ *vs. Control*) but not in the nuclear fraction (*NS*). In the cytosol, the increased intensity of high molecular weight (150-250 kDa) OGT bands was attenuated by NBt50, NBt100, and NAe ($p<0.05$ *vs. Control*).

(iii) Enrichment of O-GlcNAc at the Z-lines was confirmed by showing the co-localization of O-GlcNAc with desmin and vinculin, two cytoskeletal proteins associated with the Z-lines in cardiomyocytes. Ischemia alone had a little effect on desmin and vinculin fluorescence. After I/R, there was a marked loss in desmin fluorescence and its association with the Z-lines was disrupted, while vinculin

localization and intensity remained relatively unchanged. NBt50 largely attenuated the structural disruption and helped to maintain the striated pattern of both desmin and vinculin and their co-localization with O-GlcNAc. By immunoblot analyses, I/R markedly reduced desmin content, particularly in the membrane fraction (>2-fold), which was prevented by NBt50, NBt100 and NAe treatments (*p<0.05 vs. Control*). There were no changes in total vinculin levels in any of the groups; however, NBt50, NBt100 and NAe blunted the I/R-induced increases in vinculin phosphorylation at Tyr(822) in both whole tissue and membrane lysates (*p<0.05 vs. Control*).

(iv) Ischemia (10 min) increased overall O-GlcNAc and UDP-HexNAc (*p<0.05 vs. Normoxia*), both of which were declined following 60 min reperfusion (*p<0.05 vs. Ischemia*). ATP was decreased with ischemia (*p<0.05 vs. Normoxia*) and was not restored after reperfusion. By MALDI-TOF, identification of groups of proteins that showed increased or decreased O-GlcNAc immunoreactivity in response to ischemia and/or reperfusion resulted in three protein hits with relatively high coverage. Two proteins of O-GlcNAc positive bands that showed increased O-GlcNAc during ischemia followed by decreases with reperfusion were identified as GP (glycogen phosphorylase b; 97 kDa) and ACO (aconitase 2; 85 kDa). Third protein in a band showing increased O-GlcNAc positivity only with reperfusion that remained elevated compared to both ischemic and normoxic hearts was identified as vinculin (125 kDa). Total levels of vinculin, GP, and ACO did not differ after ischemia or reperfusion. By immunoprecipitation, all three proteins were positive with anti-O-GlcNAc antibody and showed consistent changes with stress conditions (i.e increased O-GlcNAc of vinculin only after reperfusion; with ischemia increased O-GlcNAc of GP and ACO that was decreased after reperfusion). NBt100 and NAe prevented the loss of O-GlcNAcylation of vinculin, GP, and ACO that occurred on I/R despite the equal total protein levels (regardless of stress conditions and treatments) in both whole tissue lysates and after immunoprecipitation. Incubation with β-N-acetylglucosamine completely competed away the anti-O-GlcNAc antibody. Moreover, vinculin, GP, and ACO were all co-immunoprecipitated with OGT. NBt100 and NAe prevented the I/R-induced loss of ACO and OGT levels in the mitochondrial fraction as well as the loss of aconitase enzyme activity (*p<0.05 vs. Control*).

3.1. (i) Glucosamine perfusions (0.05, 0.1, 1.0, 5.0, and 10.0 mM) had no effect on cardiac functions of RPP, ± dp/dt, and coronary flow, or ATP levels at any concentration (*NS vs. 0 mM*). Glucosamine augmented both cardiac UDP-GlcNAc and

O-GlcNAc levels at all concentrations (*$p<0.05$ vs. 0 mM*) in a clear dose-dependent manner. As low as 0.1 mM glucosamine significantly increased both UDP-GlcNAc and O-GlcNAc levels by 40-50% (*$p<0.05$ vs. 0 mM*). Glucosamine significantly increased palmitate oxidation at all concentrations (*$p<0.05$ vs. 0 mM*) with a concomitant decrease in total carbohydrate oxidation (*$p<0.05$ vs. 0 mM*) that reached a maximal response at 0.1 mM glucosamine concentration. This increase in palmitate oxidation (46±4 vs. 67±2) was associated with significantly decreased lactate (30±3 vs. 14±1) and pyruvate (8±2 vs. 3±1) oxidation (*$p<0.05$ vs. 0 mM*). Higher concentrations of glucosamine had no additional effect on substrate utilization; however, UDP-GlcNAc and O-GlcNAc levels reached a maximal (~2-fold) increase at 1 mM glucosamine concentration and were not increased further. Glucose oxidation remained unaltered at all glucosamine concentrations (*NS vs. 0 mM*). Consistent with the ~2-fold decrease in lactate oxidation, exogenous lactate uptake rates were reduced by ~2-fold with 0.1 mM glucosamine (*$p<0.05$ vs. 0 mM*). 0.1 mM glucosamine had no effect on the rate of glycolytic lactate efflux (*NS vs. 0 mM*).

(ii) Glucosamine in the heart had no effect on AMPK and ACC phosphorylation at any concentration, not even at 0.1 mM glucosamine concentration, at which palmitate oxidation was markedly enhanced. In contrast, glucosamine treatments (0.1, 1.0, 5.0, and 10.0 mM) markedly increased membrane-associated FAT/CD36 levels in a dose-dependent manner (*$p<0.05$ vs. 0 mM*).

(iii) FAT/CD36 was immunoprecipitated in whole tissue and membrane fraction of hearts in which O-GlcNAc was increased (~3 fold) with glucosamine and NBt (OGA inhibitor) at the time of 60 min normoxic perfusion. We found evidence of O-GlcNAc modification of FAT/CD36, particularly in the membrane fraction, and that OGT co-immunoprecipitated with FAT/CD36.

3.2. (i) For all studied patients, autonomic score correlated with T2DM duration (r=0.270, *$p<0.05$*). Vibration threshold values were inversely correlated with fructosamine levels (r= -0.317, *$p<0.05$*) and autonomic score values (r= -0.195, *$p<0.05$*), but not with hemoglobin A_{1c} (r= -0.179, p=0.172) or T2DM duration (r= -0.027, p=0.330).

PF-PPS infusions were well-tolerated and adverse effects or infusions-related complications were not recorded. PF-PPS decreased the autonomic score in the Verum group (3.8±0.2 vs. 2.8±0.2; *$p<0.001$*). The number of those patients that showed mild (n=36), explicit (n=37) or severe (n=3) dysfunctions prior to therapy was

markedly decreased after PF-PPS infusions, and autonomic score became normalized in 20 patients (none category) (*p<0.001*). PF-PPS improved heart rate variability to deep breath test in the Verum group (13.9±1.5 vs. 18.2±1.7; *p<0.001*). The number of patients with abnormal heart rate variability (n=47) was decreased by 23.3% after therapy (n=29) (*p<0.001*). PF-PPS increased the diastolic blood pressure rise to handgrip test in the Verum group (10±1 vs. 14±1 (mmHg); *p<0.001*). The number of patients showing abnormal response to handgrip before therapy (n=48) was decreased by 29.9% after therapy (n=25) (*p<0.001*). Other CA-N tests (Valsalva, 30/15 ratio, postural blood pressure fall) remained unaltered after PF-PPS infusions. In the Placebo group there were no changes in any of the autonomic responses after sham-infusions. Before infusions, there were no differences in patient distributions showing abnormal reflexes between the groups [(Verum/Placebo) deep breath test: 47/9; Valsalva ratio: 5/1; 30/15 ratio: 1/0; postural hypotension: 9/3; handgrip test: 48/4]. Neither were differences between the groups before (pB) nor after (pA) therapy in heart rate variability (pB=0.543; pA=0.063), Valsalva ratio (pB=0.805; pA=0.227), 30/15 ratio (pB=0.339; pA=0.718), postural hypotension (pB=0.199; pA=0.052), diastolic blood pressure rise to handgrip (pB=0.227; pA=0.087). While before therapy autonomic score did not differ between Verum and Placebo groups (3.8±0.2 vs. 3.9±0.5; pB=0.673), after therapy it was significantly lower in the Verum group (2.8±0.2 vs. 4.5±0.5; *pA<0.001 vs. Placebo*).

PF-PPS increased vibration threshold values in the Verum group (10.5±0.4 vs. 11.9±0.4; *p<0.001*). In the Placebo group, there were no changes in vibration threshold values after sham-infusions (NS; 11.6±1.2 vs. 10.7±1.2). There were no differences in vibration threshold values between Verum and Placebo groups before or after therapy at either lower limbs (right: pB=0.157, pA=0.571; left: pB=0.272, pA=0.671).

(ii) The majority of patients had normalbuminuria in both Verum (n=47; 70%) and Placebo groups (n=9; 82%). In the Verum group, ACR (mg/mM) did not change with PF-PPS infusions (3.5±1.4 vs. 4.3±1.3; p=0.364). There were also no changes in ACR in the Placebo group after sham-infusions (6.39±4.71 vs. 9.34±8.22; p=0.436). Neither were differences between the two groups before (pB=0.449) or after (pA=0.278) therapy.

3.3. (i) There were no differences in serum EPO, gender, age, lipid profile, and blood pressure between any of the groups (i.e. DM, DM+CKD, CKD, and CONTR). There was no difference in renal impairment between the DM+CKD and CKD groups (serum creatinine: p=0.795; GFR: p=0.820). There was also no difference in glycemic parameters between the DM and DM+CKD groups (fructosamine: p=0.797; hemoglobin A_{1c}: p=0.298). T2DM duration was higher in the DM+CKD group than in the DM group (20±3 vs. 13±2 years, $p<0.05$). ACE-I drugs were equally applied in all patient groups (DM, DM+CKD, and CKD).

Despite comparable serum EPO levels, hematocrit and hemoglobin were significantly lower in the DM, DM+CKD, and CKD groups (*$p<0.05$ vs. CONTR*). Patients in the DM+CKD group had lower hematocrit and hemoglobin values compared to the DM group ($p<0.05$) that was matched for glucose metabolism, and the CKD group ($p<0.05$) that was matched for renal impairment. Red blood cell properties were in the normal range with no differences between any of the study cohorts. Correlation analyses showed no significant relationships between serum EPO levels and the hematocrit [DM: R^2=0.025, p=0.575; DM+CKD: R^2=0.106, p=0.236; CKD: R^2=0.000, p=0.949] or the hemoglobin values [DM: R^2=0.000, p=0.956; DM+CKD: R^2=0.100, p=0.316; CKD: R^2=0.000, p=0.960] in any of the patient groups. Correction analyses examined for several variables (e.g. age, serum creatinine, hemoglobin A_{1c}, fructosamine, serum glucose, total and HDL cholesterol, and blood pressure) showed significant relationships between serum EPO levels and hematocrit values only in the DM group after corrections for body mass index (R^2=0.410, $p<0.05$) and triglyceride levels (R^2=0.285, $p<0.05$).

(ii) In a subgroup of anemic patients with low baseline serum EPO levels, administration of 1 gram ASA significantly increased the serum EPO levels by 59% (7.71±5.41 vs. 12.26±8.73), the reticulocyte count by 33% (42±12 vs. 56±16), the reticulocyte ratio by 14% (0.81±0.49 vs. 0.92±0.68), the red blood cell count by 7% (3.75±0.26 vs. 4.01±0.29), the hemoglobin by 6% (111.1±7.6 vs. 118.3±8.6), and the hematocrit values by 8% (33.0±2.6 vs. 35.8±2.6) ($p<0.01$). At the same time, ASA significantly decreased LDH by 12% (319±36 vs. 280±35; $p<0.01$).

DISCUSSION

1. (i) We showed that acute exposure of endothelial cells to CSB increased eNOS phosphorylations resulting in shift to an inhibitory state as well as the disruption of enzimatically active eNOS dimers. These findings support the notion that cigarette smoke reduces eNOS activity, thus NO bioavailability, which is an important factor in the progression of macro- and microvascular diseases. The notion that CSB increased eNOS phosphorylations at both the activating Ser(1177) and inhibitory Thr(495) residues seems contrary to a common concept that there is a simultaneous reciprocal phosphorylation-dephosphorylation reaction at these two sites. Alternatively, there may be independent upstream regulatory responses to CSB leading to increased eNOS phosphorylations of both sites.

(ii) We showed that GSH, a potent free radical and aldehyde scavenger salvaged eNOS from inactivating modifications by preventing its inhibitory phosphorylation and the disruption of homodimeric eNOS into an inactive, mainly superoxide producing monomeric form. Thus, GSH could be beneficial to preserve eNOS activity and NO production, presumably by neutralizing the aldehydes (especially the formaldehyde), which are abundant in CSB (Mazák et al.). Protective effects of GSH suggest that aldehydes may have a role in mediating the CSB-induced oxidant stress leading to increased eNOS phosphorylations and that homodimeric eNOS structure can be conserved by abolishing the oxidative damage of key thiol groups within eNOS.

(iii) Several protein kinases have been identified to regulate eNOS activity in response to different stimuli. For example, both PKA and PKB/Akt could activate eNOS via Ser(1177) phosphorylation under physiological conditions (e.g. shear stress, bradykinin) leading to increased enzyme activity and NO production. We showed that CSB concentration- and time-dependently inactivated PKB/Akt resulting in dissimilar changes of decreased phospho-Ser(473) Akt and increased phospho-Ser(1177) eNOS levels, and this latter was further increased with selective PI3-K inhibitor treatment. These findings indicate that PI3-K/PKB/Akt pathway could not be responsible for directly phosphorylating eNOS at Ser(1177) in response to CSB. Selective PKA inhibitor (H-89) to CSB did not alter phospho-eNOS levels at either Ser(1177) or Thr(495) site, thus acute effects of CSB on eNOS phosphorylations seemed to be also independent of the PKA pathway.

(iv) The ubiquitous PKC superfamily has a wide array of actions in signal transduction and, to date, it has been shown to maintain eNOS phosphorylation at

Thr(495) only in unstimulated endothelial cells. Activation of PKC pathway, in particular PKCβII, has been implicated in mediating the adverse effects of diabetes on vascular-endothelial function; whereas ruboxistaurin (PKCßII-inhibitor) attenuated vascular dysfunctions and preserved endothelium-dependent vasodilation. We showed that PKC pathway blockade (Ro-318425) markedly suppressed phospho-Thr(495) eNOS levels to CSB and that ruboxistaurin recapitulated the effects seen with selective PKC inhibitor by decreasing phospho-Thr(495) eNOS levels in a concentration-dependent manner. Taken together, activation of PKC pathway, more specifically PKCβII appears to play a key role in mediating the CSB-induced eNOS phosphorylations by increasing its inhibitory phosphorylation at Thr(495), rather than its activating phosphorylation at Ser(1177). PKCßII inhibition with ruboxistaurin could be a promising strategy to prevent the cigarette smoke induced deleterious effects.

2. (i) We showed that NAG-thiazolines at the time of reperfusion, which compared to PUGNAc have markedly higher selectivity for OGA, protected the isolated heart against I/R injury as indicated by increased functional recovery, decreased post-ischemic arrhythmic activity and tissue injury. Both NBt and NAe markedly increased cardiac O-GlcNAc in a drug- and dose-dependent manner, and the degree of protection was directly proportional to the effectiveness of NAG-thiazolines at increasing O-GlcNAc (i.e. NAe > NBt at equivalent concentrations). NAG-thiazolines had no effect on ATP and UDP-HexNAc levels, thus improved functional recovery and attenuated tissue injury during reperfusion was clearly not a consequence of improved bioenergetic status or increased flux through the HBP. The significant correlations between increased O-GlcNAc, improved functional recovery, and reduced tissue injury indicated that protection was related to the specificity of OGA inhibitor, supporting the notion that inhibiting OGA with NAG-thiazolines exerts cardioprotective effects via increased protein O-GlcNAcylation. Cardioprotection was seen even though NAG-thiazolines were given only at the time of reperfusion; and improved functional recovery was evident after as early as 5 min of reperfusion. This might be of clinical relevance in settings where very early revascularization treatment protocols are practical. Increasing O-GlcNAc by inhibiting OGA with NAG-thiazolines during reperfusion may be a valuable cardioprotective strategy; however, their potential utility against I/R injury *in vivo* has yet to be determined.

(ii) Immunohistochemistry of normoxic hearts showed higher O-GlcNAc in the nuclei, consistent with other systems; as well as a clear cross-striated pattern in the

cytosol corresponding to Z-lines in cardiomyocytes. We first showed that cardiac proteins at the Z-lines are highly enriched in O-GlcNAc, which given the importance of Z-lines in regulating cardiomyocyte function by mediating hemodynamic and mechanical responses, could have important implications for protein O-GlcNAcylation in the heart beyond its cardioprotective effects. Both ischemia alone and I/R resulted in a striking loss of O-GlcNAc within the nuclei showing perinuclear O-GlcNAc staining and punctate, which were not seen in the normoxic hearts. We first showed that one consequence of ischemia and I/R is a redistribution of nuclear and cytoplasmic O-GlcNAc in the heart. While OGA inhibition significantly attenuated the I/R-induced loss of O-GlcNAc and the structural dysintegrity (i.e. disorganized myofibrils, loss of cross-striated O-GlcNAc), it did not prevent the ischemia and I/R induced loss of nuclear O-GlcNAcylation (i.e. O-GlcNAc negative nuclei). Immunoblot analyses also revealed that recovery of nuclear O-GlcNAc levels lagged behind the cytosolic levels in both NBt and NAe hearts, which may simply reflect the fact that OGA is predominantly (~90%) localized in the cytosol.

(iii) We showed the co-localization of O-GlcNAc with desmin and vinculin, two cytoskeletal proteins associated with the Z-lines in cardiomyocytes. We demonstrated that OGA inhibition during reperfusion prevented the loss of desmin and helped to maintain its association with the Z-lines. Following I/R, both NBt and NAe largely attenuated the loss of desmin in whole tissue and particularly in the membrane fraction. Preservation of desmin, which has a key role in contractile force transmission, structural integrity and linking mechanical stresses to the nucleus, could contribute to the improved functional recovery and reduced tissue injury seen with OGA inhibitors. In contrast to earlier reports showing a progressive loss and/or altered subcellular localization of vinculin with I/R, we found no changes in vinculin levels, and although there were some focal alterations in vinculin fluorescence (e.g. dislocated Z-lines, higher intensity at intercalated discs), the co-localization of vinculin to the Z-lines and intercalated discs remained relatively conserved. OGA-inhibitors, however, attenuated the I/R-induced increases in vinculin Tyr(822) phosphorylation. Physiological consequences of vinculin phosphorylation in the heart are unclear. Disruption of interacting proteins (e.g. vinculin) at the focal adhesion complexes has been shown to exacerbate ischemic injury in cardiomyocytes. Thus, it is possible that decreased vinculin phosphorylation seen with OGA inhibitors could help to prevent the disruption of focal adhesion complexes thus the structural dysorganization after I/R. We also

identified vinculin as a potential O-GlcNAc target and being associated with OGT. As to how O-GlcNAcylation/phosphorylation of vinculin alters the function of the heart in response to I/R has yet to be determined.

(iv) We showed that although ischemia alone, after as little as 10 min, increased overall O-GlcNAc, there was a marked loss in O-GlcNAcylation after I/R, which appears contrary to earlier reports indicating that increasing cellular O-GlcNAc was an endogenous response to stress (Zachara et al.). The mechanisms underlying the loss of O-GlcNAc following I/R are not known. Fülöp et al. reported that ischemia increased O-GlcNAc of the perfused heart followed by a decline during reperfusion. Nőt et al. observed similar phenomenon *in vivo* after hemorrhagic shock, where the loss of tissue O-GlcNAcylation was sustained for up to 24 hours following resuscitation. H_2O_2 treatment of cardiomyocytes resulted in the loss of O-GlcNAcylation, which was attenuated with the OGA inhibitor PUGNAc (Jones et al.). This suggests that oxidative stress, which is related to conditions such as reperfusion, resuscitation or exposure to H_2O_2, could be involved in mitigated O-GlcNAcylation. I/R markedly decreased active OGT (110 kDa) in whole tissue lysates and all subcellular fractions (i.e. nuclear, mitochondrial, cytosolic), of which in the cytosolic fraction high molecular weight OGT immunoreactive bands (150-250 kDa) were apparent. NBt and NAe not only prevented the loss of active OGT, but also decreased the intensity of these bands, which could be inactive aggregates or multimers of OGT. However, presence of a band at ~140-150 kDa suggests a posttranslational modification, possibly arising from increased ROS damage to OGT.

We showed that ischemia and/or I/R caused altered O-GlcNAc modification of some target proteins, such as GP, ACO2, and vinculin; and their O-GlcNAcylated levels could be augmented with NAG-thiazolines. However, we do not know the precise mechanism by which O-GlcNAcylation of either protein could contribute to cardioprotection. Given the large number of proteins subjected to O-GlcNAcylation, it is unlikely that O-GlcNAc modification of a single protein could account for the cardioprotective effects.

Cardioprotective mechanisms associated with NAG-thiazolines and increased O-GlcNAcylation have yet to be determined. Functional recovery was enhanced by NBt and NAe within the first 5-10 min of reperfusion, suggesting the role of transcriptionally independent mechanisms. Reperfusion injury involves impaired Ca^{2+}-homeostasis, increased oxidative stress, and mitochondrial dysfunction. Augmented O-GlcNAc of

the intact heart attenuated Ca^{2+}-overload to Ca^{2+}-paradox as well as decreased the activity of Ca^{2+}-sensitive proteases following I/R (Liu et al.). NAG-thiazolines prevented the loss of desmin during reperfusion, which is very susceptible to Ca^{2+}-activated proteases thus preservation of desmin could be a consequence of its decreased proteolysis. Reduced arrhythmogenesis and improved LVDP seen with NBt and NAe could be a result of normalized cytosolic and mitochondrial Ca^{2+} levels, respectively. Increased O-GlcNAc protected the cardiomyocytes against lethal ROS damage via increased mitochondrial stability (Jones et al.). NBt in cardiomyocytes reduced the apoptotic and necrotic cell death following I/R, and attenuated the H_2O_2-induced loss of mitochondrial membrane potential (Champattanachai et al.). We showed that NBt and NAe at the time of reperfusion prevented the loss of aconitase activity, which is known to be sensitively inactivated by ROS. NBt and NAe at the time of reperfusion also increased the mitochondrial levels of aconitase and its association with O-GlcNAc and OGT. Thus, it is likely that myocardial preservation seen with NAG-thiazolines in response to I/R could be due to reduced Ca^{2+}-overload, decreased oxidative stress or both. Links between O-GlcNAcylation and oxidative stress suggest that O-GlcNAc signaling may represent an important redox-sensing pathway that could play integral roles in the ROS-induced cellular responses thus modulating stress tolerance of the heart to I/R injury.

3.1. (i) We showed that acute (60 min) activation of the HBP with glucosamine, which dose-dependently increased UDP-GlcNAc and O-GlcNAc of the isolated heart, resulted in a significant increase in fatty acid oxidation with a concomitant decrease in total carbohydrate oxidation, similarly to that seen in the diabetic heart. Decreased total carbohydrate oxidation was due primarily to reduced lactate oxidation and a lesser extent to pyruvate oxidation, while glucose oxidation was unchanged. This shift in cardiac metabolism was very similar to that Wang et al. reported in ZDF-rat hearts perfused under similar conditions, where the onset of T2DM was associated with increased fatty acid and decreased carbohydrate oxidation (mainly the lactate) accompanied by impaired cardiac contractility; although we did not see impaired cardiac functions here, due possibly to short-term treatment (60 min). Glucosamine had no effect on glucose-derived lactate efflux (i.e. glycolysis), indicating that glucosamine in the heart at least, is not metabolized via glycolysis and TCA cycle. Effects of glucosamine on substrate utilization were apparent at relatively low concentrations (maximal at 0.1 mM), at which both UDP-GlcNAc and O-GlcNAc were

markedly increased, suggesting that fairly subtle changes in the HBP flux and O-GlcNAc turnover could play an important role in the regulation of cardiac metabolism. Even though higher glucosamine levels (1-10 mM) had no further effect on substrate utilization, there was a progressive increase in UDP-GlcNAc and O-GlcNAc, consistent with HBP being the primary pathway for glucosamine metabolism in the heart.

(ii) In contrast to adipocytes (Luo et al.), glucosamine had no effect on AMPK or ACC activation in the heart that could account for the increased palmitate oxidation. Dissimilar changes can be explained by differences in treatments (sustained-24 hours vs. acute-60 min), and/or the tissue specificity of AMPK regulation (adipose vs. cardiac tissue). Although this does not exclude the possibility that more sustained activation of the HBP and O-GlcNAcylation increases AMPK and ACC activity, which plays a crucial role in energy and metabolic homeostasis in the heart. Nevertheless, we showed that glucosamine dose-dependently increased membrane-associated FAT/CD36, which is responsible for ~50-80% of fatty acid uptake in the heart thus facilitating fatty acid oxidation. Thus, increased palmitate oxidation by glucosamine may be, at least in part, a consequence of increased FAT/CD36. There was a dissociation between increased FAT/CD36 levels that continued to increase up to 5-10 mM glucosamine (similar that seen with O-GlcNAc) and palmitate oxidation that showed a maximal increase at 0.1 mM glucosamine, which is consistent with excess fatty acid uptake being channeled primarily to esterification rather than oxidation.

(iii) In diabetes, increased FAT/CD36 expression has been linked to increased myocardial fatty acid transport and oxidation, as well as the impairment of its recycling between intracellular storage compartments and the sarcolemma. FAT/CD36 appeared as a target for O-GlcNAc modification, in particular in the membrane fraction, and was also associated with OGT. It is possible that glucosamine induced increases in membrane-associated FAT/CD36 may be mediated by increasing O-GlcNAcylation of FAT/CD36, possibly via blocking FAT/CD36 recycling and shifting the balance toward its sarcolemmal localization. In conclusion, effects of glucosamine on myocardial substrate utilization (i.e. increased palmitate oxidation) and increased membrane levels of FAT/CD36 are likely to be mediated via increased HBP flux and O-GlcNAcylation. Sustained activation of these pathways in diabetes could be an alternative mechanism contributing to the pathogenesis of diabetic cardiomyopathy.

3.2. (i) We showed that combination of PF-PPS, two pro-circulatory and anti-inflammatory drugs, significantly improved CA-N and vibration perception in T2DM

patients. At baseline, indicated by increased autonomic score, a greater part of study participants exhibited CA-N, including severe (53%) and mild (40%) cases. CA-N correlated with T2DM duration and impaired vibration perception, consistent with the notion that autonomic neuropathies generally occur after longer duration of diabetes and that CA-N often coexists with other peripheral neuropathies. CA-N affected both parasympathetic (i.e. impaired heart rate variability to deep breath, 61%) and sympathetic functions (i.e. abnormal handgrip test, 62%), both of which were improved by PF-PPS. Other CA-N tests, most probably due to low prevalence (Valsalva test: 7%, 30/15 ratio: 1%, or orthostatic hypotension: 12%) were not influenced. Nevertheless, PF-PPS significantly decreased the autonomic score indicative of an overall improvement and increased the number of Verum group patients showing mild or normal CA-N tests. Vibration threshold values inversely correlated with fructosamine, indicating adverse effects of `brief` hyperglycemic episodes on large sensory nerve function. PF-PPS improved vibration threshold values, while there were no changes in Placebo group after sham-infusions. In accordance, patients in the Verum group reported significant improvements of their symptoms upon surveys, such as burning pain or numbness, while these remained unaltered in sham-treated patients, although we did not use visual analog scale. It is acknowledged that specific mechanisms by which PF-PPS improved CA-N and vibration perception have not been examined; however, both pro-circulatory and anti-inflammatory/oxidant properties of PF-PPS could have important contributions. Short-term administration of PF-PPS (5 days iv.) in regular intervals may be a complementary and cost-effective therapeutic strategy to improve CA-N and peripheral sensory neuropathy, mostly among hospitalized T2DM patients.

(ii) While we found that most of the study participants showed CA-N (93%) and in a lesser degree impaired vibration perception (35%), the majority of patients had normalbuminuria (72%), most likely due to an increased use of RAS-inhibitors. PF-PPS had no effect on urinary albumin excretion rate, which is in contrast to earlier reports demonstrating that PF markedly reduced proteinuria when added on to ACE-I and/or ARB therapy or when administered alone in patients with T2DM nephropathy. However, most of these studies used long-term PF treatments at relatively high doses (400-1200 mg) and in patients with more advanced stages of nephropathy (i.e. micro-, and macroalbuminuria). It is likely that anti-proteinuric effect of PF could not be afforded within the normal range, or the short duration (5 days) and/or low dose of PF

(100 mg) may be also insufficient to convey reduction in albuminuria. In future, randomised, well-designed, multicentre studies are warranted for evidence-based recommendation of PF and PPS in the management of diabetic microangiopathy.

3.3. (i) We showed that unresponsiveness to endogenous EPO occurs in patients with T2DM and/or CKD leading to lowered hematocrit and hemoglobin values. Correlation analyses of EPO with either hematocrit or hemoglobin showed no relationships in any of the patient groups, indicating indeed the presence of EPO resistance in T2DM and CKD. Co-existence of both conditions, T2DM and CKD (i.e. diabetic nephropathy) induced the highest degree of EPO resistance and normocyter anemia, which was unrelated to deficiencies (e.g. iron, folic acid or vitamin B_{12}). This is consistent with the notion that hypoproliferative anemia occurs earlier and it is more severe in diabetic CKD than in non-diabetic CKD contributing to the higher risk of ischemic organ damage. We found significant relationships between serum EPO and hematocrit levels in T2DM patients after corrections for BMI and triglyceride levels, suggesting that hypertriglyceridemia and obesity, both of which are closely related to insulin resistance and increased inflammatory markers and cytokines (e.g. CRP, TNF-α) may convey EPO resistance.

(ii) We showed that ASA, an anti-inflammatory and hydroxyl free radical scavenger, significantly increased serum EPO levels and ameliorated anemia in patients with T2DM and CKD, supporting the notion that pro-inflammatory processes and oxidative stress most likely contribute to the loss and/or ineffectiveness of endogenous EPO. ASA significantly increased EPO levels in such a short-term (48 hours), and resultant time-dependent increases in red blood cell count (7%) lagged behind the increases in reticulocyte number (33%); moreover, this was accompanied by decreased LDH (12%). An inflammatory response due to low EPO levels, known as neocytolysis causes the selective destruction of young erythrocytes (neocytes) leading to decreased red blood cell count and increased LDH. Conclusively, administration of ASA via its anti-inflammatory effects and subsequent acute increases of EPO together could inhibit neocytolysis and salvage the young, circulating red blood cells (`neocytosalvation`). These results also indicate that neocytolysis (due to low EPO and increased inflammation) represents an alternative mechanism being responsible for anemia in patients with T2DM and CKD.

Ph.D. THESES

1) Acute effects of cigarette smoke result in endothelial dysfunction via altered posttranslational eNOS modifications in endothelial cells.

- CSB increases the inhibitory eNOS phosphorylation at Thr(495) and the level of catalytically inactive eNOS monomers, both of which could contribute to a reduced NO availability.
- GSH diminishes the CSB-induced inactivating eNOS modifications by preventing its inhibitory phosphorylation and the disruption of homodimeric eNOS, thus contributing to preserved eNOS activity and NO production.
- Acute increases of eNOS phosphorylations to CSB appear to be independently regulated by the PI3-K/Akt pathway, whereas the PKC/PKCβII pathway seems responsible for the increased inhibitory phosphorylation at Thr(495). PKCßII inhibition could be a promising strategy to prevent the adverse effects of cigarette smoke.

2) Augmentation of protein O-GlcNAcylation with NAG-thiazolines is an effective cardioprotective strategy against ischemia-reperfusion (I/R) injury of the isolated heart.

- Selective inhibition of OGA at the time of reperfusion by NAG-thiazolines improves functional recovery and attenuates tissue injury of the isolated heart in an O-GlcNAc dependent manner.
- Reperfusion injury associated with oxidative stress decreases O-GlcNAc and OGT levels in the heart, both of which could be prevented by NAG-thiazolines.
- Myocardial proteins within the nuclei and at the Z-line regions are highly enriched in O-GlcNAc; and one consequence of ischemia and I/R is the redistribution of nuclear and cytoplasmic O-GlcNAc-associated proteins in the heart.
- Selective inhibition of OGA at the time of reperfusion by NAG-thiazolines preserves myocardial integrity and attenuates the I/R-induced changes of Z-line proteins in an O-GlcNAc dependent manner.

3) Activation of the hexosamine biosynthesis pathway (HBP) and protein O-GlcNAcylation represents a novel mechanism for the regulation of cardiac metabolism.

- Activation of the HBP and protein O-GlcNAcylation with glucosamine in the intact heart results in increased fatty acid utilization and decreased carbohydrate oxidation, similarly to that seen in the diabetic heart.
- Altered substrate utilization with glucosamine in the heart is not related to the activation of AMPK and ACC.
- Increased fatty acid oxidation appears to be a consequence of increased membrane-associated FAT/CD36 levels possibly via increased O-GlcNAcylation of FAT/CD36.

4) Combined pentoxifylline (PF) and pentosan polysulfate (PPS) infusion therapy is an effective approach to improve cardiovascular autonomic neuropathy (CA-N) and peripheral sensory neuropathy in patients with T2DM.

- Short-time infusion therapy with PF and PPS, two pro-circulatory and anti-inflammatory drugs, improves the CA-N and vibration perception in T2DM patients.
- PF-PPS in turn has no effect on albuminuria within the normal range.

5) Acetylsalicylic acid (ASA) increases serum EPO levels and ameliorates anemia in patients with T2DM and chronic kidney disease.

- Both T2DM and CKD induce EPO resistance, and there is a higher degree of EPO resistance and anemia when both conditions are present in diabetic nephropathy.
- Treatment with the anti-inflammatory and hydroxyl free radical scavenger ASA corrects the low EPO levels and anemia of patients with T2DM and CKD, at least in part, by inhibiting the rate of neocytolysis.

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(* authors contributed equally to works)

Cumulative impact factor of listed publications related to the Ph.D. thesis: 16.753; cumulative impact factor of all publications (without abstracts): 33.782

Cumulative impact factor of listed abstracts related to the Ph.D. thesis: 39.349; cumulative impact factor of all abstracts (without publications): 78.866

ACKNOWLEDGEMENTS

I would like to express my appreciation to **Prof. Dr. István Wittmann** and **Prof. Dr. Judit Nagy** for providing me the opportunity to be a member of a great scientific research group and clinicians at the 2nd Department of Internal Medicine and Nephrological Center, and for supporting my scientific carrier and clinical work since I had graduated. Without their excellent leadership and encouragement I would not have been able to finish my Ph.D.

I am very grateful to **Prof. Dr. John C. Chatham** for giving me the chance to work at the Division of Cardiovascular Disease at UAB in a very pleasant environment, for helping me in my scientific progress and I do appreciate his exceptional enthusiasm, productive and inspiring suggestions that advanced my scientific thinking and carrier.

I am very thankful to **Dr. László Wagner**, **Dr. István Mazák**, **Dr. Zoltán Wagner** for their continued suggestions during my postdoc years and support in my work.

Special thanks to **Dr. Gergő A. Molnár**, who helped a lot when I was a 'beginner', and thanks to my former postdoc-mates **Dr. Mónika Tamaskó**, **Dr. Lajos Markó**, **Dr. Judit Cseh**, **Dr. Márton Mohás** for their contributions to common projects, for sharing joyful moments and successes in the lab.

I am very thankful to **Anikó L. Heitmanné** and **Ilona V. Sámikné**, for teaching me first the basic rules around a lab and how to work precisely, for their excellent technical assistance and for their great, 'mother-like' friendship.

I am very pleased to have friends and colleagues like **Enikő Bodor** and **Zsuzsanna Rumszauer**, who have been always kindly helping me out in anything that seems `mission impossible`, many thanks for their administrative and organizing contributions to my official affairs.

I am especially thankful to **Charlye A. Brocks**, who showed me first what Southern hospitality is all about, for making my professional life smooth and my private life so enjoyable in Birmingham, she has been always a true and caring friend to me. Certainly, I would not have been able to manage my works without her exceptional technical assistance and amity.

I am also thankful to my friends and co-authors **Dr. Sue A. Marsh** for her constructive suggestions and critics, and for her valuable contributions to my works, to **Dr. Norbert Fülöp** for teaching me heart perfusions and for his thoughtful inputs to my works, to **Dr. László G. Nőt** for having been always there for me in my professional and personal life.

I would like also thank to all my colleagues in the laboratory of **Prof. Dr. Richard B. Marchase** and **Prof. John. C. Chatham**, to **Erica Taylor**, **Xiaoyuan Zhu**, **Luyun Zou**, **Dan Shan**, **Vorarrat Champattanachai**, **Kai He**, **Chris Calderon** for their great support and for being my friends during my stay in Birmingham.

I would like to also thank **Prof. John C. Chatham** for having my work appreciated and providing me the opportunity to collaborate with principal investigators and members of acclaimed research groups. Here I would like to acknowledge the help of **Prof. Dr. Richard B. Marchase** (President of the Federation of American Societies for Experimental Biology), **Prof. Dr. Suzanne Oparil** (President of the American Society of Hypertension), **Prof. Dr. Victor Darley-Usmar** (President of the Society for Free Radical Biology and Medicine), **Prof. Dr. Christine Des Rosiers** (Montreal Heart Institute, Université de Montréal), **Prof. Martin E. Young D.Phil.** (Division of Cardiovascular Disease, UAB), **Dr**. **James A. Mobley** (Director of Bioanalytical and Mass Spectrometry Facility, UAB), **Prof. Dr. Andrew J Paterson** (Division of Endocrinology, Diabetes and Metabolism, UAB), **Prof. Dr. Roger C. White** (Division of Cardiovascular Disease, UAB) during our grateful collaboration, and the assistance of my co-authors **Elena Ulasova**, **Arzu Onay-Besikci**, **Bradford G. Hill**, **Kai Wang,** and **Landon Wilson**.

I am thankful for **Everyone, Friends and Colleagues** who are not listed here, but had contributed to my accomplishments in my professional or personal life.

And at last but not least I would like to thank my **Family**, without their continous support and compassionate love I would not have been capable of achieving my goals and dreams!