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Antioxidant treatment attenuates lactate production in diabetic nephropathy

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ABSTRACT

The early progression of diabetic nephropathy is notoriously difficult to detect and quantify prior to the occurrence of substantial histological damage. Recently, hyperpolarized [1-^{13}C] pyruvate has demonstrated increased lactate production in the kidney early after the onset of diabetes, implying increased lactate dehydrogenase activity as a consequence of increased nicotinamide adenine dinucleotide substrate availability due to upregulation of the polyol pathway, i.e., pseudohypoxia. In this study, we investigated the role of oxidative stress in mediating these metabolic alterations using state-of-the-art hyperpolarized magnetic resonance (MR) imaging.

Ten-week-old female Wistar rats were randomly divided into three groups: healthy controls, untreated diabetic (streptozotocin treatment to induce insulinopenic diabetes), and diabetic, receiving chronic antioxidant treatment with TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) via the drinking water. Examinations were performed 2, 3, and 4 weeks after the induction of diabetes by using a 3T Clinical MR system equipped with a dual tuned ^{13}C/^{1}H-volume rat coil. The rats received intravenous hyperpolarized [1-^{13}C] pyruvate and were imaged using a slice-selective ^{13}C-IDEAL spiral sequence.

Untreated diabetic rats showed increased renal lactate production compared to that shown by the controls. However, chronic TEMPOL treatment significantly attenuated diabetes-induced lactate production. No significant effects of diabetes or TEMPOL were observed on ^{13}C-alanine levels, indicating an intact glucose-alanine cycle, or ^{13}C-bicarbonate, indicating normal flux through the Krebs cycle.

In conclusion, this study demonstrates that diabetes-induced pseudohypoxia, as indicated by an increased lactate-to-pyruvate ratio, is significantly attenuated by antioxidant treatment. This demonstrates a pivotal role of oxidative stress for renal metabolic alterations occurring in early diabetes.

INTRODUCTION

Patients diagnosed with developing diabetic nephropathy are left with current treatments that at best slow disease progression (19). Thus, effective prevention and treatment of diabetic nephropathy requires a better understanding of the mechanisms involved in progressive kidney damage. Today, microalbuminuria is the preferred marker of diabetic nephropathy, but by the time microalbuminuria can be detected, abnormal renal structural changes have already occurred. Hyperpolarized ^{13}C-labeled pyruvate was recently introduced as a possible alternative endogenous marker for the identification of renal metabolic alterations in diabetic rats. This technique allows for real-time repeated measurements of intrarenal metabolism during the development of kidney disease (5-7). Prolonged hyperglycemia causes altered oxygen metabolism via an increased flux through the polyol pathway, leading to imbalanced NADH/NAD^+ distribution, which in turn increases lactate formation even though sufficient oxygen is present for oxidative phosphorylation to occur. Such a metabolic alteration is commonly referred to as pseudohypoxia (25). However, despite the name, pseudohypoxia will eventually result in the development of "true" renal hypoxia as the disease progresses (3, 15). Thus, the balance between pseudohypoxic and hypoxic conditions has been proposed to be an indication of disease progression (5-7).

Oxidative stress has been reported to be a crucial component of diabetic nephropathy, and antioxidant treatment in animal models of diabetic nephropathy has generally resulted in reduction or even complete prevention of disease progression (24). The relationship between increased oxidative stress and metabolic alterations in the diabetic kidney has not been established, although a close relationship between metabolic alteration and development of diabetic nephropathy has been reported (15). Therefore, we tested the hypothesis that chronic antioxidant treatment prevents pseudohypoxia in a rat model of insulinopenic diabetes using hyperpolarized metabolic imaging.

MATERIALS AND METHODS

Twenty-four ten-week-old female Wistar rats (Taconic, Ry, Denmark) were included in this study. The rats were randomly grouped into an untreated diabetic group (n=14), a 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL)-treated diabetic group (n=8), and a healthy control group (n=6). Diabetes was induced by an intravenous injection of freshly prepared streptozotocin (STZ; 55 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 10 mmol/L cold citrate buffer (pH 4.5). Blood glucose was measured in tail-capillary blood with a Contour XT blood glucose meter (Bayer Diabetes Care, Copenhagen, Denmark). Rats were considered diabetic if blood glucose levels exceeded 15 mmol/L at 48h after STZ injection. In order to identify the development of diabetic complications, a longitudinal within-subject study design was used for the analysis of the development of diabetic nephropathy. This part of the study was performed on healthy (N=6) and N=10 (data from two rats were excluded from the diabetic group due to erroneous MRI acquisitions and only a single time point of data acquisition, resulting in (N=8)) animals with up to three examinations per animal. The examinations were performed at two, three, and four weeks after diabetes induction (Fig. 1A). In order to identify the response to TEMPOL treatment a parallel group was investigated at only one time point consisting of untreated diabetic (N=4, test of difference with within-subject diabetic animals) and TEMPOL treated diabetic animals (N=8) at the terminal point (Fig. 1B). No difference was found between the longitudinal and the single time point diabetic groups at the terminal point. The study complied with the Guidelines for Use and Care of Laboratory Animals and was approved by the Danish Inspectorate of Animal Experiments.

Tail vein catheterization was performed for hyperpolarized [1-^{13}C] pyruvate administration, and then the animals were placed in a clinical magnetic resonance (MR) scanner for metabolic imaging. Temperature, oxygen saturation, and respiration were monitored throughout the MR imaging

session (SA Instruments, Stony Brook, NY, USA). After the final MR imaging (MRI) session, a blood sample was collected from the aortic bifurcation using ethylenediaminetetraacetic acid (EDTA) coated tubes. MRI sessions were performed on a 3T HDx clinical scanner (GE Healthcare, Waukesha, WI, USA) equipped with a dual tuned $^{1}H/^{13}C$-volume transmit/receive rat radio frequency coil (GE Healthcare, Waukesha, WI, USA). The kidneys were localized by a standard localization sequence. Axial, coronal, and oblique ^{1}H-T_2 weighted fast spin echo experiments were used for metabolic overlay and anatomical information. The sequence parameters were: matrix=384x384 (interpolated to 512x512), field of view (FOV)=80x80 mm^2, flip angle=90°, repetition time (TR)=3000 ms, number of transients=16, number of echoes=28, echo time (TE)=104, 3-7 slices of 5 mm thickness, covering both kidneys in axial, coronal, and oblique orientations. A slice-selective ^{13}C-IDEAL spiral sequence was used for hyperpolarized [1-^{13}C] pyruvate imaging, acquiring images every 5 s and initiated 20 s after the start of injection. Sequence parameters were as follows: a flip angle of 10º, 11 IDEAL echoes and one initial spectrum per IDEAL encoding, TR/TE/ΔTE=100 ms/0.9 ms/0.9 ms, FOV=80x80 mm^2, 5x5 mm real resolution and an axial slice thickness of 15 mm covering both kidneys.

Hyperpolarization

A solution of 127 mg of [1-^{13}C] pyruvic acid (Cambridge Isotope Laboratories, Tewksbury, MA) mixed with 15 mM AH111501 (GE Healthcare, Brøndby, DK) was polarized in a 5T SPINlab (GE Healthcare, Brøndby, DK), to a reproducible polarization of more than 40% with a final concentration of 125 mM.

MRI Analysis

Data analysis of the hyperpolarized MR signal was processed using a vendor delivered Matlab (MathWorks, Natick, MA, USA) package, and the raw DICOM images were transferred to OsiriX (Pixmeo, Geneva, Switzerland) for anatomical overlay and region of interest (ROI) analysis. The metabolite signal was normalized relative to the pyruvate signal in each individual kidney.

Quantitative real-time PCR

After euthanasia, kidney cortices were rapidly dissected and snap-frozen in liquid nitrogen. Cortical tissue was homogenized and total RNA was isolated using a Nucleospin RNA II kit (Stratagene; AH Diagnostics, Aarhus, DK) following the manufacturer's instructions. RNA purity and concentration was measured spectrophotometrically (Eppendorf Biophotometer, Hørsholm, Denmark). cDNA synthesis was performed on 0.5 µg RNA with a revert first strand cDNA synthesis kit (Thermo Scientific, Hvidovre, DK) according to the manufacturer's instructions. Preparation of samples for qPCR was performed using Maxima SYBR Green qPCR master mix (Thermo Scientific, Hvidovre, DK), following the manufacturer's instructions. The qPCR protocol consisted of 40 cycles of: denaturation for 30 seconds at 95°C followed by annealing and synthesis for 1 min at 60°C, and was performed in a 96-well qPCR plate using an AriaMx PCR system (Agilent Technology, Glostrup, Denmark). Primer information for each individual gene is presented in *Table 1.*

Biochemical assays and ELISA for KIM-1 and NGAL

Fumarate, lactate and succinate assays (Sigma-Aldrich) and ELISA for Kidney injury marker 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) ELISA (Abcam, Cambridge, UK) were performed according to the manufacturer's instructions In brief, kidney cortex tissue was

snap-frozen in liquid nitrogen. Thereafter, tissue was homogenized in assay buffer specific for each assay; the homogenate centrifuged, and assays and ELISA performed on the resultant supernatant.

Blood was collected from the abdominal aorta, transferred to heparin tubes and centrifuged to isolate the plasma fraction. All samples were stored at -80◦C. Analysis was performed in 96-well Costar plates in a PHERAstar FS micro plate reader (BMG Labtech, Birkerød, Denmark). The full absorbance spectrum was determined for each wavelength in order to optimize each assay.

Statistics

Normality was assessed with quantile plots. Two-sided $p<0.05$ was considered statistically significant. A multivariate repeated measure analysis of variance (ANOVA) based on the mixed model approach to allow missing values was used to compare the metabolic response as a function of time and performed using STATA (StataCorp LP, TX, USA). No additional *post hoc* corrections were performed. If the mean response (within-subjects, between-subject, and interaction) was significantly different, a pairwise comparison of the individual time points was performed.

The group differences at the terminal point (Fig. 1B) to investigate the treatment response to TEMPOL and the biochemical analysis were performed using a one-way-ANOVA with Fisher *post hoc* correction using GraphPad, Prism (GraphPad Inc. La Jolla, CA, USA).

RESULTS

Administration of STZ resulted in hyperglycemia within 48h, and blood glucose levels remained significantly elevated throughout the study period (*p<0.0001*; Fig. 2). Diabetic animals developed pronounced renal hypertrophy (*p<0.0001*), while total body weight did not significantly differ between the groups (*p=0.3*; Fig. 2). Chronic TEMPOL treatment to diabetic animals did not significantly affect any of these parameters compared to the untreated diabetic group (Fig. 2).

The lactate-to-pyruvate ratio was significantly elevated in the diabetic kidney at three weeks (+38% versus healthy controls; *p=0.01*) and further increased (+77% versus healthy control; *p=0.001*) at four weeks after the induction of diabetes (Fig. 3, Table 2). No significant time-dependent changes between healthy controls and diabetic rats were observed for either alanine-to-pyruvate or bicarbonate-to-pyruvate ratios, although a significant overall time dependency was found in the bicarbonate-to-pyruvate ratio (Fig. 3, Table 2). Chronic TEMPOL treatment significantly reduced lactate production (*p=0.03*) compared to untreated diabetic rats, but had no effect on alanine (*p=0.47*) or bicarbonate levels (*p=0.11*; Fig. 4).

The mRNA expression of lactate dehydrogenase was significantly elevated in the diabetic group compared to healthy controls (*p=0.01*). Chronic TEMPOL treatment significantly attenuated this increase, as treated animals presented a similar expression level as controls (*p=0.5*; *Fig. 5*). mRNA expression of pyruvate dehydrogenase (*p=0.09*) and alanine transferase (*p=0.2*) was similar in all groups (Fig. 5). No significant differences were observed in mRNA expression of oxygen-sensitive genes, kidney injury markers, or inflammation markers (Fig. 6). Chronic TEMPOL treatment significantly decreased cortical tissue lactate concentration in both controls (*p=0.046*) and diabetics (*p=0.0025*), while a significantly increased lactate concentration was seen in plasma compared to untreated controls (*p=0.013*). The concentration of the TCA intermediate fumarate was similarly significantly reduced by chronic TEMPOL treatment in cortical tissue compare to controls

(*p=0.002*) and diabetic (*p=0.031*) animals. Succinate concentrations, was significantly increased in both diabetic animals with (*p=0.001*) and without (*p<0.001*) chronic TEMPOL treatment. No significant differences were observed in either succinate or fumarate concentrations in plasma (Fig. 7). No significant different concentrations of NGAL (*p=0.072*) or KIM-1 concentration (*p=0.095*) between controls and diabetic animals with and without chronic TEMPOL treatment (Fig. 8).

DISCUSSION

The main finding of this study was that antioxidant treatment with TEMPOL attenuated diabetes-induced lactate formation, and thus improved the lactate-to-pyruvate ratio in diabetic animals. This finding implies improved metabolic status secondary to reduced pseudohypoxia and supports the use of hyperpolarized [1-^{13}C] pyruvate as a biomarker for renal metabolic status under different treatment regimens. TEMPOL is a potent antioxidant known to prevent oxidative stress and to improve insulin sensitivity (2). Interestingly, we have recently shown that suboptimal insulin treatment does not improve the lactate-to-pyruvate ratio (5), indicating that the main mechanism for the phenomenon seen in TEMPOL-treated animals is likely due to direct antioxidant effects.

The diabetic kidney suffers from altered metabolism, manifesting as increased total oxygen consumption secondary to increased mitochondrial leak respiration and decreased tubular electrolyte transport efficiency (4). Furthermore, increased metabolic flux via the polyol pathway causing altered cellular NADH/NAD$^+$ ratio results in increased lactate production and increased lactate-to-pyruvate ratio despite sufficient oxygen availability for oxidative phosphorylation (3). This condition is therefore commonly referred to as “pseudohypoxia.” (25). It has been demonstrated that the inhibition of aldose reductase, the initial step in the polyol pathway, effectively prevents increased lactate formation and the subsequent reduction of the interstitial pH in the diabetic kidney (15). Indeed, most data supporting an altered NADH/NAD$^+$ ratio originate from *in vitro* experiments (13) due to the methodological difficulties in obtaining reliable and reproducible results from *in vivo* approaches. The present study demonstrates, using a non-invasive imaging method that only requires an intravenous injection of the tracer, a novel link between oxidative stress and pseudohypoxia in the diabetic kidney. This provides an additional mechanistic explanation for the beneficial effects of antioxidant treatment on diabetic kidney function (14, 17).

The lactate-to-pyruvate ratio depends on the degree of diabetes-induced kidney damage, whereas alanine transferase and pyruvate dehydrogenase activity seems to be largely unaffected. This is in agreement with our previous findings, which reported increased intrarenal lactate-to-pyruvate ratios in insulinopenic rats (5-7). Furthermore, a time-dependent accumulation of lactate in the diabetic kidney has been previously reported as well (8). The decreased cortical lactate concentration in response to TEMPOL treatment is in good agreement with the findings using hyperpolarized [1-^{13}C]pyruvate. It has previously been reported that increased succinate concentrations stimulate renin release (21). Succinate is converted by succinate dehydrogenase into fumarate in the TCA cycle and we therefore investigated the pool size of fumarate. An inverse relationship between fumarate and succinate was observed. This is contrary to previous studies reporting significantly elevated fumarate concentrations in another animal model of experimental diabetes (26). It can be speculated that varying severity of diabetes and different animal models may influence these findings. However, it was previously demonstrated that treatment with an NOX1/NOX4 inhibitor decreased fumarate levels, implying a pivotal involvement of oxidative stress. This is in agreement with the results from the present study demonstrating that the antioxidant TEMPOL significantly alters this metabolic pathway. However, it should be noted that PDH flux was unaltered and that leak respiration provides a substantial contribution to total kidney oxygen consumption in diabetes (17). Necrosis and subsequent cellular leak could be a potential mechanism for increased extracellular concentrations of TCA metabolites (21,26). Although the lactate-to-pyruvate ratio was significantly increased in the diabetic kidney, the mRNA expression of oxygen-sensitive and renal dysregulation genes were not altered, suggesting an earlier identification of the renal abnormalities observed using hyperpolarized MR.

Biomarkers for kidney injury, such as NGAL and KIM1, have been demonstrated to correlate with loss of kidney function in patients with type-2 diabetes (12). Elevated levels of these kidney injury

markers have also been reported in experimental models of insulinopenic diabetes (1,22,23). However, KIM1 is not an independent predictor for progression of diabetic nephropathy in patients with type 1 diabetes when adjusted for albumin excretion rate (16). Given the available data, NGAL and KIM1 might provide better prediction of kidney outcome in the setting of acute kidney injury compared to the more chronic setting of diabetic nephropathy. Although the results from the present study tend to imply increased NGAL and KIM1 in diabetic rats chronically treated with TEMPOL, it is unclear how this translates to long-term outcome given the lack of solid literature support for a relationship between these biomarkers and progression of diabetic nephropathy. Indeed, previous reports have demonstrated a time-dependent regulation of these genes (9, 18). The efficiency of the antioxidant treatment is most likely time-dependent, i.e. earlier treatment initiation after the onset of diabetes leads to more potent effects. This may further explain why clinical trials investigating the use of antioxidants as a strategy to treat ongoing diabetic nephropathy have failed (20). It should also be noted that clinically available substances are relatively weak antioxidants compared with TEMPOL, which may contribute to the ineffective outcomes of these trails (10).

The ability to assess lactate-to-pyruvate conversion non-invasively within seconds, in combination with standard anatomical and functional MR measurements, and the recent introduction of hyperpolarized MR in human subjects (11) argue in favor of the introduction of this modality for the assessment and therapeutic monitoring of diabetic nephropathy, facilitating the introduction of valuable novel metabolic information for clinicians.

Summary and Significance

Pseudohypoxia has classically been considered resultant of an altered NADH/NAD^+ ratio secondary to increased metabolic flux via the polyol pathway. However, the present study demonstrated that antioxidant treatment of insulinopenic rats with TEMPOL significantly reduced the lactate-to-

pyruvate ratio, which is an indication of reduced pseudohypoxia. A link between increased diabetes-induced oxidative stress and pseudohypoxia in the kidney has so far not been established. These results provide an additional mechanistic explanation for the beneficial effects of reduced oxidative stress for diabetic kidney function. However, the finding of greater metabolic abnormalities with disease progression indicates that interventions are likely to be more effective if initiated early on after the onset of diabetes. Furthermore, the results demonstrate that the use of the lactate-to-pyruvate ratio as a biomarker of disease development is far more sensitive compared to classical markers such as NGAL and KIM-1. An early and sensitive biomarker for diabetic nephropathy would allow for early interventional studies, which are urgently needed since no effective treatment strategy to fight disease progression, outside minimizing known risk factors, is currently available.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

All authors participated in the design and interpretation of the study. C.L. researched data and wrote the manuscript draft. P.M.N., H.Q., T.S.N., U.K, and L.B.B. researched data. H.S.J., J.A.Ø., A.F., and J.H.A. reviewed/edited the manuscript. F.P. contributed to discussion and wrote/edited the

manuscript. C.L. is the guarantor of this work and, as such had full access to all the data of the study and takes responsibility for the integrity and the accuracy of the data analysis.

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Figure legends

Figure 1. Study design, consisting of up to three consecutive hyperpolarized MRI examinations in a healthy and untreated diabetic group, and the inclusion of untreated diabetic animals and a third group treated for three weeks with TEMPOL examined at the 4-week endpoint.

Figure 2. End-point blood glucose levels, kidney weights, and body weights at the end of study in control (N=6) and diabetic animals with and without chronic TEMPOL treatment (N=10). *denotes $p<0.05$ between controls versus untreated and treated diabetes, respectively. The data are shown as mean±SEM.

Figure 3. Lactate-to-pyruvate, alanine-to-pyruvate, and bicarbonate-to-pyruvate levels in healthy controls (N=5) and untreated diabetic rats (N=10) during the first week after the onset of insulinopenic diabetes. *denotes $p<0.05$ in subject as a function of time and between controls and diabetic animals. The data are shown as mean±SEM. (**Lactate**) $p=0.03$* for group (*between subjects factor),* $p=0.02$ for time (*within-subjects factor)* and $p=0.01$ for interaction; (**Alanine**) $p=0.66$ for group, $p=0.46$ for time and $p=0.81$ for interaction; (**Bicarbonate**) $p=0.98$ for group, $p=0.02$ for time and $p=0.50$ for interaction.

Figure 4. Endpoint lactate-to-pyruvate, alanine-to-pyruvate, and bicarbonate-to-pyruvate ratios in control (N=4) and diabetic animals (N=11) with and without chronic TEMPOL treatment (N=8). *denotes $p<0.05$ between diabetics versus controls and diabetics treated with TEMPOL. **denotes $p<0.05$ between controls and diabetic treated with TEMPOL. The data are shown as mean±SEM.

Figure 5. mRNA expressions of lactate dehydrogenase, alanine transferase, and pyruvate dehydrogenase in control (N=5) and diabetic animals (N=13) with and without chronic TEMPOL treatment (N=8). *denotes $p<0.05$ between diabetics versus controls and diabetics treated with TEMPOL. The data are shown as mean±SEM.

Figure 6. mRNA expressions of oxygen-sensitive genes ((NAD(P)H quinone acceptor oxidoreductase 1 (*NQO1*), Vascular endothelial growth factor (*VEGF*), heme oxygenase-1 (*HO1*)), and markers of kidney injury (*KIM-1* and *NGAL*), and fibrosis ((α-Smooth Muscle Actin (*ACTA2*)) and inflammation (tumor necrosis factor α (*TNFα*)) in control (N=5) and diabetic animals (N=13) with and without chronic TEMPOL treatment (N=8). The data are shown as mean±SEM.

Figure 7. Tissue and plasma concentrations of lactate and the TCA metabolites fumarate and succinate in control (N=6) and in diabetic animals (N=14) with and without chronic TEMPOL treatment (N=8). The data are shown as mean±SEM.

Figure 8. Cortical tissue NGAL and KIM-1 protein concentration in control (N=6) and in diabetic animals (N=14) with and without chronic TEMPOL treatment (N=8). The data are shown as mean±SEM.

Table 1. PCR primers.

Gene	Forward primer sequence	Reverse primer sequence
ACTB	5′-AGCCATGTACGTAGCCATCC-3′	5′-TGTGGTGGTGAAGCTGTAGC-3′
LDH	5′-AATATTACGTGAAATGTAAGAT-3′	5′-TTTTCCTTGGCATGACACTTGAG-3′
PDH	5′-TCCACTCCTTGTAGCTGCAAC-3′	5′-GAGAACCCACCACCCCATG-3′
ALT	5′-GCCATGTATTCCTTCCCTCA-3′	5′-GCCTCATTGAAGACCTGCTC-3′
KIM-1	5′-CCACAAGGCCCACAACTATT-3′	5′-TGTCACAGTGCCATTCCAGT-3′
NGAL	5′-GATCAGAACATTCGTTCCAA-3′	5′-TTGCACATCGTAGCTCTGTA-3′
HO1	5′-TCTATCGTGCTCGCATGAAC-3′	5′-AAGGCGGTCTTAGCCTCTTC-3′
VEGF	5′-GCCCATGAAGTGGTGAAGTT-3′	5′-ACTCCAGGGCTTCATCATTG-3′
ACTA2	5′-CATCATGCGTCTGGACTTGG-3′	5′-CCAGGGAAGAAGAGGAAGCA-3′
TNFα	5′-GCCCTAAGGACACCCCTGAGGGAGC-3′	5′-TCCAAAGTAGACCTGCCCGCACTCC-3′
NQO1	5′-GTGGTGATGGAAAGCAAGGT-3′	5′-GCCCGGATATTGTAGCTGAA-3′