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BRIEF COMMUNICATION

Effect of Prediabetes on Membrane Bicarbonate Transporters in Testis and Epididymis

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Abstract The formation of competent spermatozoa is a complex event that depends on the establishment of adequate environments throughout the male reproductive tract. This includes the control of bicarbonate (HCO_3^-) concentration, which plays an essential role in the maintenance of extracellular and intracellular pH (pH_i) values. Diabetes mellitus alters pH_i regulation in mammalian cells, mainly by altering the activity of ion transporters, particularly HCO_3^--dependent mechanisms. Yet, little is known about the effects of this pathology and its prodromal stage, prediabetes, on the membrane transport mechanisms of male reproductive tract cells. Herein, we analyzed protein and mRNA levels of the most relevant HCO_3^- transporters of the SLC4 family [anion exchanger 2 (AE2), Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE), electrogenic Na^+/HCO_3^- cotransporter 1 (NBCe1), electroneutral Na^+/HCO_3^- cotransporter 1 (NBCn1)] in the testis and epididymis of a prediabetic animal model. Firstly, we identified the HCO_3^- transporters of the SLC4 family, in both testicular and epididymal tissue. Secondly, although no alterations were detected in protein expression, mRNA levels of NBCe1, NBCn1 and NDCBE were significantly increased in the testis of prediabetic rats. On the other hand, in the epididymis, prediabetes caused an increase of AE2 and a decrease of NDCBE protein levels. These alterations may be translated into changes of HCO_3^- transepithelial epididymal fluxes in vivo, which may represent a threat for sperm survival. Moreover, these results provide evidence of the molecular mechanism that may be responsible for the significant increase in abnormal sperm morphology already reported in prediabetic rats.

Keywords Bicarbonate transport · Male fertility · Membrane transporter · SLC4 · Prediabetic state

Abbreviations

AE2	Anion exchanger 2
BTB	Blood–testis barrier
DM	Diabetes mellitus
HED	High energy diet
NBCe1	Electrogenic Na^+/HCO_3^- cotransporters
NBCn1	Electroneutral Na^+/HCO_3^- cotansporters
NDCBE	Na^+ driven Cl^-/HCO_3^- exchanger
pH_i	Intracellular pH
qPCR	Real-time PCR
SLC4	Solute carrier 4
STF	Seminiferous tubular fluid
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

Introduction

All functions of the organism require the maintenance of an adequate intracellular pH (pH_i), which generally is strictly kept within a narrow range (Boron 2004; Jones et al. 1995). The regulation of pH_i largely depends upon the activity of plasma membrane carriers that mediate the transport of acid/base equivalents (Madshus 1988). Cells possess in their plasmatic membrane a wide range of ion transporters that participate in pH_i regulation, among which are the

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basic and acidic particle membrane transporters (Boron 2004). Among these, the solute carrier 4 (SLC4) gene family encodes several widely distributed membrane proteins that facilitate the exchange of Cl^-, HCO_3^-, Na^+ and borate across the plasma membrane of mammalian cells, which contribute decisively to the regulation of pH_i, cell volume and membrane potential (Alper 2006).

Within the male reproductive tract, the formation of competent spermatozoa is a complex process that involves the production of a large number of germ cells in the testis and several maturation steps that occur in the excurrent ducts. During the entire process, establishment of adequate environments throughout the male reproductive tract is vital for the production and maturation of spermatozoa and for providing a means of transport. A central feature of the luminal fluid is its pH (Pastor-Soler et al. 2005). HCO_3^- concentration is a major player that helps to maintain this parameter within suitable values in order to promote the appropriate progression of spermatozoa development. In mammals, the pH of luminal fluids of the reproductive system has significant effects on male reproductive potential (Pastor-Soler et al. 2005). In fact, disturbances in acid–base balance on the reproductive tract have been reported as a cause for male subfertility or infertility (Breton et al. 1996).

Instability in pH_i has been reported in several pathological conditions, resulting from either altered cellular metabolism and/or cellular and subcellular membrane changes, such as those that are known to occur in diabetic conditions (Feuvray 1997). Diabetes mellitus (DM) is one of the most prominent public health threats in modern societies, and its prevalence is drastically increasing. Actually, the World Health Organization estimates that there will be at least 300 million people living with the disease in 2025 (WHO 2002). The vast majority of diagnosed DM cases are classified as type 1 (T1DM) or type 2 (T2DM). The complexity of DM diagnosis, especially in obese patients, led the investigators to establish an intermediate state, often called the "prediabetic" state. This prodromal state is characterized by resistance to insulin-mediated glucose disposal and compensatory hyperinsulinemia (Alves et al. 2013; Reed et al. 2000). Prediabetic patients have major metabolic disorders that increase the risk for T2DM development (Engelgau et al. 2000). Furthermore, the incidence of DM has been closely linked with the decreasing fertility rates of modern societies (Lutz 2006), especially due to the growing occurrence of DM in men of reproductive age.

With this short communication we present clear evidence for the effect of the prediabetic state on the expression of specific HCO_3^--dependent membrane ion transporters, particularly those of the SLC4 family, in testicular and epididymal tissues. This will provide new insights on how the progression of DM might interfere with pH regulation, inducing male reproductive dysfunction.

Materials and Methods

Animal Model

Ten 2-month-old male Wistar rats were used in the present study. The animals were housed in our accredited animal colony (Health Sciences Research Centre, University of Beira Interior) and maintained with food and water ad libitum at a constant room temperature (20 ± 2 °C) on a 12-h cycle of artificial lighting. Rats were randomly divided into control and high-energy-diet (HED) groups. Control animals were fed a standard chow diet (4RF21 certificate; Mucedola, Settimo Milanese, Italy), and the HED group received an additional high-energy emulsion as described elsewhere (Rato et al. 2013). After the treatment, animals were killed by cervical dislocation. Testes and epididymis were removed and processed for experiments or stored at −80 °C. All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication 85-23, revised 1996) and the European directive for the care and handling of laboratory animals (Directive 86/609/EEC).

As described by Rato et al. (2013), HED administration to adult rats led to the development of two main characteristics, glucose intolerance and mild hyperglycemia, suggesting that HED animals developed a prediabetic state, in which some, but not all, of the diagnostic criteria for diabetes are met.

Quantitative RT-PCR

Real-time quantitative PCR was performed to evaluate the mRNA expression of anion exchanger 2 (AE2), electrogenic Na^+/HCO_3^- cotransporters (NBCe1), electroneutral Na^+/HCO_3^- cotransporters (NBCn1) and Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE) as previously described (Vaz et al. 2012). Specific primers were designed for amplification of the target and housekeeping transcripts (Table 1). β_2-Microglobulin transcript levels were used to normalize gene expression levels. Fold variation of gene expression levels was calculated following the mathematical model proposed by Pfaffl (2001), using the formula $2^{-\Delta\Delta Ct}$.

Western Blot

Western blotting was performed as previously described by Alves et al. (2011). Membranes were incubated with goat anti-SLC4A4 (1:250, Sc-162214, Santa Cruz Biotechnology,

Table 1 Genes, oligonucleotide sequence and respective conditions for PCR amplification of AE2, NDCBE, NBCn1, NBCe1 and β_2-Microglobulin

Gene	Sequence (5′–3′)	AT (°C)	Amplicon (bp)	Cycles
AE2	Sense: ATGCCAAAGGGTCTACACAG	53	138	35
NM_017048.2	Antisense: GCTCCTGGTTTTTGTCCAAC			
NDCBE	Sense: GAGACCTACCCCATCCACAT	53	189	35
NM_199497.2	Antisense: TATGAACTCCCCGTGCATCT			
NBCn1	Sense: GATGAAATGGCCAAAACTGCC	53	107	35
NM_058211.2	Antisense: ATTGTCACACTCACAGGCTT			
NBCe1	Sense: GCCTGGAGAACAACCAAAGT	53	131	35
NM_053424	Antisense: CATACAGAACAGGCATGGGG			
β_2-Microglobulin	Sense: ATGAGTATGCCTGCCGTGTG	58	92	30
NM_012512.2	Antisense: CAAACCTCCATGATGCTGCTTAC			

AT annealing temperature

Inc.), goat anti-AE2 (1:250, Sc-46710, Santa Cruz Biotechnology, Inc.), goat anti- SLC4A8 (1:500, Sc-169346, Santa Cruz Biotechnology, Inc.) or rabbit anti-SLC4A7 (1:250, Sc-99633, Santa Cruz Biotechnology, Inc.). Mouse anti-α-tubulin (1:5,000, A5441, Sigma-Aldrich) was used as a protein loading control. Immunoreactive proteins were detected separately with donkey anti-goat IgG-AP (1:5,000, Sc-2020, Santa Cruz Biotechnology, Inc.), goat anti-rabbit IgG-AP (1:5,000, Sc-2004, Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG-AP (1:5,000, Sc-2005, Santa Cruz Biotechnology, Inc.). Membranes were reacted with the ECF detection system. The densities from each band were obtained using Quantity One Software (Bio-Rad, Hemel Hempstead, UK), divided by the respective α-tubulin band density and then normalized against the respective control.

Statistical Analysis

The statistical significance of the sample variation among the experimental groups was assessed by one-way ANOVA. The results are presented as relative variation in comparison with the control group. All experimental data are shown as mean ± SEM ($n = 5$ for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $p < 0.05$ was considered significant.

Results

Prediabetes Causes Significant Increases in mRNA Levels of NBCe1, NBCn1 and NDCBE in Testis

The effect of HED on mRNA transcript levels of AE2 was evaluated by quantitative PCR. The mRNA expression of AE2 in testis was not significantly altered when compared with the control group (0.92 ± 0.15-fold variation). Protein expression levels confirmed that HED had no effect on AE2 compared to the control group (1.03 ± 0.17-fold variation) (Fig. 1). Protein levels of NBCe1 were not significantly different in the testis from HED-treated rats compared with the control group (0.81 ± 0.19-fold variation). In contrast, the mRNA expression levels of NBCe1 in the testis of HED-treated rats presented a significant increase of 2.94 ± 0.21-fold relative to the control group (Fig. 1).

The mRNA expression of the electroneutral transporter NBCn1 was significantly increased in testis of HED-treated rats (4.22 ± 0.17-fold increased to control). However, this was not followed by an increase in protein expression levels of these transporters (0.811 ± 0.190-fold variation relative to control) (Fig. 1).

Evaluation of mRNA NDCBE levels in the testis of HED-treated rats showed a significant increase of 2.00 ± 0.31-fold relative to the control group. However, when we evaluated the protein levels of this HCO_3^- transporter, no significant alterations were observed in the testis of HED-treated rats compared to the control group (Fig. 1).

Prediabetes Causes a Significant Increase in Protein Levels of AE2 and a Decrease in Protein Levels of NDCBE in Epididymis

In the epididymis of HED-treated rats, mRNA expression levels of AE2 were not significantly different from those found in the epididymis of rats from the control group (0.64 ± 0.08-fold variation) (Fig. 2). However, when we evaluated the protein expression levels of this transporter, there was a significant increase of AE2 protein expression in the epididymis of HED-treated animals relative to the control (1.77 ± 0.50-fold variation) (Fig. 2).

In the epididymis of HED-treated rats, the mRNA expression of NBCe1 was decreased compared with the

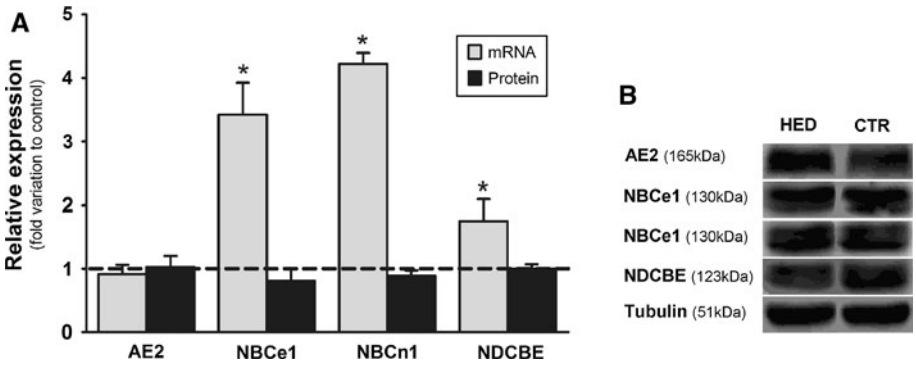

Fig. 1 Effect of high-energy diet (HED) on membrane transporters: anion exchanger 2 (*AE2*), electrogenic Na^+/HCO_3^- cotransporters (*NBCe1*), electroneutral Na^+/HCO_3^- cotransporters (*NBCn1*) and Na^+-driven Cl^-/HCO_3^- exchanger (*NDCBE*), mRNA and protein levels in rat testes. **a** Pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in testis tissue of HED rats compared with testis from control condition (*dashed line*). **b** Illustrative Western blot experiment. Results are expressed as mean ± SEM ($n = 5$). *Significantly different relative to control ($p < 0.05$)

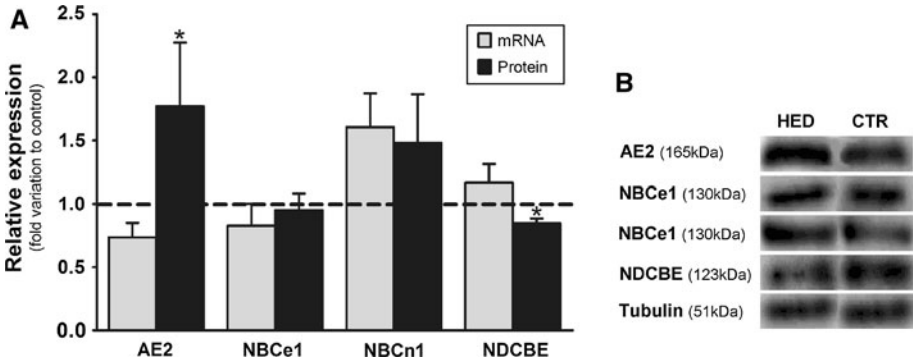

Fig. 2 Effect of high-energy diet (HED) on membrane transporters: anion exchanger 2 (*AE2*), electrogenic Na^+/HCO_3^- cotransporters (*NBCe1*), electroneutral Na^+/HCO_3^- cotransporters (*NBCn1*) and Na^+-driven Cl^-/HCO_3^- exchanger (*NDCBE*), mRNA and protein levels in rat epididymis. **a** Pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in epididymal tissue of HED rats compared with epididymis from control animals (*dashed line*). **b** Illustrative Western blot experiment. Results are expressed as mean ± SEM ($n = 5$). *Significantly different relative to control ($p < 0.05$)

control group (0.71 ± 0.15-fold variation); however, this alteration was not statistically significant. Likewise, the protein levels of this transporter were not significantly altered in the epididymis of rats from the HED group (0.95 ± 0.13-fold variation relative to control) (Fig. 2).

Similarly, the mRNA and protein levels of NBCn1 in the epididymis were not significantly different in HED-treated rats compared with the control group. Both mRNA and protein levels from the epididymis of HED-treated rats presented a nonsignificant increase to 1.606 ± 0.265- and 1.817 ± 0.270-fold variation relative to the control group, respectively (Fig. 2).

Finally, although HED-treated animals did not present a significant alteration in epididymal NDCBE mRNA transcript levels (1.16 ± 0.15-fold variation vs. control), the protein expression levels of this transporter were significantly decreased in the epididymis of HED-treated rats (0.85 ± 0.04-fold decrease relative to control) (Fig. 2).

Discussion

It has been reported that DM does not change steady-state pH_i but significantly alters pH_i regulation in mammalian cells, mainly by markedly decreasing the activity of some ion transporters (Feuvray 1997). It has also been suggested that some HCO_3^--dependent mechanisms of pH regulation may be depressed in cells of DM individuals (Khandoudi et al. 1995). Nevertheless, little is known about the effects of DM, or of any of the prodromal stages of the disease such as prediabetes, on the membrane transport mechanisms involved in pH regulation throughout the male reproductive tract cells. Hence, we induced a prediabetic state in male rats following ingestion of a HED, as previously described by our team (Rato et al. 2013), and evaluated the effect of this prodromal stage of DM on the expression of the most relevant HCO_3^- transporters of the SLC4 family: AE2 (SLC4A2), NBCe1 (SLC4A4), NBCn1 (SLC4A7) and NDCBE (SLC4A8).

The two-compartment configuration of the testis (the seminiferous tubules and the intertubular areas), filled with the characteristic fluids [seminiferous tubular fluid (STF) and testicular lymph or interstitial fluid] (Jegou et al. 1982), is of great relevance for its functioning. For instance, it has been reported that the composition of the fluid within the seminiferous tubules is very stable due to the existence of the blood–testis barrier (BTB). Furthermore, the luminal milieu is markedly distinct from the interstitial fluid and plasma, and these differences are critical to the normal occurrence of spermatogenesis (Rato et al. 2010).

A study by Caflisch and DuBose (1990) showed that rat STF contains a very low concentration of HCO_3^-. These results led to the assumption that active HCO_3^- secretion is not an important factor in the formation of STF. Nevertheless, it has been described that Sertoli cells, which are responsible for STF secretion, express several HCO_3^- membrane transporters involved in pH_i regulation (Oliveira et al. 2009a, b).

In the testis, the presence of all the HCO_3^- transporters of the SLC4 family studied had already been described (for review, see Bernardino et al. 2013). AE2 mRNA expression was reported in the various testicular somatic cells (Sertoli, peritubular and Leydig cells), in developing germ cells and in "mature" spermatozoa (Holappa et al. 1999). Indeed, studies on knockout mice have shown that AE2 plays an essential role in spermatogenesis (Liu et al. 2012). NBCe1 expression has also been detected in the testis, although its subcellular localization has not been discussed (Liu et al. 2011). Expression of NDCBE has been reported at very high levels in the testis (Boron 2001; Grichtchenko et al. 2001) and particularly in Sertoli cells (Oliveira et al. 2009a, b). It has been suggested that NDCBE might play an important role in maintaining Sertoli cell pH_i homeostasis (Oliveira et al. 2009a, b). The presence of electroneutral NBCn1, usually located in the basolateral membrane of epithelial cells (Boron et al. 2009), has also been reported in the testis. In testicular tissue, NCBn1 mRNA expression was detected based on microarray and expressed sequence tag studies (Uhlen et al. 2010). Herein we confirmed the expression of all the HCO_3^- transporters of the SLC4 family studied at the testicular level, both at the mRNA and at the protein level. Although no alterations were detected in protein expression, the mRNA levels of NBCe1, NBCn1 and NDCBE were significantly increased. Even though, as previously observed, the protein levels do not always reflect the changes in mRNA transcript levels and, hence, the changes in mRNA level are not a direct measure of the variation in the protein quantity or functioning, they can be a clear indication of the effectiveness of the regulation exerted on the studied protein. The diminished mRNA levels not reflected in protein changes could be explained by differential rates of synthesis or degradation or both. Moreover, mRNA half-lives can increase or decrease in response to a variety of stimuli including hormones and growth factors (Hollams et al. 2002). Thus, the possibility exists that prediabetes modulation of the analyzed mRNA quantities in the testis is exerted at the transcriptional and/or post-transcriptional level and/or that the modulation of protein quantities is regulated by other mechanisms or occurs in a different time frame.

On the other hand, at the epididymal level prediabetes was able to alter the protein expression of several HCO_3^- transporters. As discussed, epididymal function is important to regulate and modify the luminal fluid content, which is crucial for male fertility, namely, for the maturation, transport, concentration and storage of spermatozoa. Both epididymal secretion and reabsorption of luminal fluids establish and modify the epididymal microenvironment (Foley 2001) in order to provide an adequate environment to transform spermatozoa into fully mature cells (Robaire et al. 2006). The fluid milieu in the epididymis is acidic, containing very low concentrations of HCO_3^- (Rodriguez-Martinez et al. 1990), pointing toward a major role of HCO_3^- membrane transport mechanisms in the establishment of luminal fluid pH and, thus, in overall epididymis function.

As at the testicular level, the presence of all the HCO_3^- transporters of the SLC4 family studied had already been described in the epididymal tissue (for review, see Bernardino et al. 2013). Expression of AE2 was reported in the epithelial cells lining the lumen of the epididymis. According to Medina et al. (2003), AE2 is exclusively localized in the basolateral membrane of cells in the male reproductive excurrent ducts, being highly expressed in relatively low abundance in the distal regions, including the cauda epididymis (Jensen et al. 1999b). Jensen and collaborators (1999a) reported that NBCe1 is expressed in the basolateral membrane of principal and apical/narrow cells of the rat epididymis. It has been proposed that NBCe1 is localized in the basolateral membrane of those cells and that, by facilitating HCO_3^- extrusion into the interstitial space, it might contribute to luminal HCO_3^- uptake by this epithelium (Pastor-Soler et al. 2005). A strong presence of NDCBE has also been detected in the epididymis (Liu et al. 2011; Uhlen et al. 2010). No data regarding the cellular distribution of this transporter in the cells of the epididymal tract are available. The presence of NBCn1 has also been clearly identified in rat epididymis, where it has been localized at the apical membrane of specialized epididymal cells (narrow cells and clear cells) (Pushkin et al. 2000).

Our results confirmed the expression of the studied HCO_3^- transporters of the SLC4 family, at both the mRNA and protein levels. The epididymis of prediabetic rats showed a significant increase in AE2 and a significant decrease in NDCBE protein levels. Expression of AE2 has been reported in the basolateral membrane of epithelial

cells lining the lumen of all the regions of the epididymis (Medina et al. 2003), and this transporter is known to be expressed in relatively low abundance in the cauda epididymis (Jensen et al. 1999b). The presence of AE2 in the basolateral membrane of epithelial cells of the proximal parts of the epididymis correlates with the low luminal concentration of HCO_3^- attained in these segments, and thus, it has been suggested that basolateral AE2 might contribute, in parallel with other HCO_3^- transporters, to net HCO_3^- reabsorption. We were able to detect a decrease of NDCBE protein levels. This transporter has been described as a pH_i regulator that transports extracellular Na^+ and HCO_3^- in exchange for intracellular Cl^- and/or H^+, playing an important role in cellular alkalinization (Russell and Boron 1976) and in HCO_3^- reabsorption.

As discussed, the establishment of a low HCO_3^- concentration in the lumen of the epididymis contributes to maintaining an optimal environment for proper sperm storage and viability. Alteration of the HCO_3^- transepithelial epididymal fluxes in vivo might, therefore, represent a real threat for sperm survival during storage in the epididymis, and this might correlate with the results described by Rato et al. (2013), who reported a significant increase in abnormal sperm morphology in prediabetic rats.

In conclusion, at the testicular level, there was an increase in the mRNA levels of NBCe1, NBCn1 and NDCBE in prediabetic rats, although no differences were observed at the protein level. On the other hand, at the epididymal level, prediabetes altered the protein expression of some of the studied HCO_3^- transporters, causing a significant increase in AE2 and a significant decrease in NDCBE protein levels. These results suggest an alteration in HCO_3^- homeodynamics in the lumen of the epididymis, which may affect the establishment of a proper environment for sperm storage and viability and, hence, male reproductive potential.

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Conflict of interest The authors confirm that this article has no conflict of interests.

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