Original Article

MOLECULAR METABOLISM

TRPC proteins contribute to development of diabetic retinopathy and regulate glyoxalase 1 activity and methylglyoxal accumulation

Robin Sachdeva [1,9], Andrea Schlotterer [2,9], Dagmar Schumacher [1,9], Christin Matka [1,9], Ilka Mathar [1], Nadine Dietrich [2], Rebekka Medert [1], Ulrich Kriebs [1], Jihong Lin [2], Peter Nawroth [3,4,5,6], Lutz Birnbaumer [7,8], Thomas Fleming [3,4], Hans-Peter Hammes [2], Marc Freichel [1,*]

ABSTRACT

Objective: Diabetic retinopathy (DR) is induced by an accumulation of reactive metabolites such as ROS, RNS, and RCS species, which were reported to modulate the activity of cation channels of the TRPC family. In this study, we use *Trpc1/4/5/6*$^{-/-}$ compound knockout mice to analyze the contribution of these TRPC proteins to diabetic retinopathy.

Methods: We used Nanostring- and qPCR-based analysis to determine mRNA levels of TRPC channels in control and diabetic retinae and retinal cell types. Chronic hyperglycemia was induced by Streptozotocin (STZ) treatment. To assess the development of diabetic retinopathy, vaso-regression, pericyte loss, and thickness of individual retinal layers were analyzed. Plasma and cellular methylglyoxal (MG) levels, as well as Glyoxalase 1 (GLO1) enzyme activity and protein expression, were measured in WT and *Trpc1/4/5/6*$^{-/-}$ cells or tissues. MG-evoked toxicity in cells of both genotypes was compared by MTT assay.

Results: We find that *Trpc1/4/5/6*$^{-/-}$ mice are protected from hyperglycemia-evoked vasoregression determined by the formation of acellular capillaries and pericyte drop-out. In addition, *Trpc1/4/5/6*$^{-/-}$ mice are resistant to the STZ-induced reduction in retinal layer thickness. The RCS metabolite methylglyoxal, which represents a key mediator for the development of diabetic retinopathy, was significantly reduced in plasma and red blood cells (RBCs) of STZ-treated *Trpc1/4/5/6*$^{-/-}$ mice compared to controls. GLO1 is the major MG detoxifying enzyme, and its activity and protein expression were significantly elevated in *Trpc1/4/5/6*-deficient cells, which led to significantly increased resistance to MG toxicity. GLO1 activity was also increased in retinal extracts from *Trpc1/4/5/6*$^{-/-}$ mice. The TRPCs investigated here are expressed at different levels in endothelial and glial cells of the retina.

Conclusion: The protective phenotype in diabetic retinopathy observed in *Trpc1/4/5/6*$^{-/-}$ mice is suggestive of a predominant action of TRPCs in Müller cells and microglia because of their central position in the retention of a proper homoeostasis of the neurovascular unit.

Keywords Diabetic retinopathy; Reactive metabolites; TRPC cation channels; MethylGlyoxal; Vasoregression; Glyoxalase1

1. INTRODUCTION

Reactive metabolites (RM), which accumulate under hyperglycemia, include reactive carbonyl (RCS), oxygen (ROS), and nitrogen (RNS) species, all of which contribute to the progression of diabetic long-term complications [1]. Such metabolites can impair the function of multiple cell types involved in diabetes-associated organ dysfunction leading to neuropathies and vasculopathies [1,2]. RM evoke post-translational modifications of numerous signaling molecules. Several types of cation channels have been identified as target molecules of such metabolites accumulating under hyperglycemia. The dicarbonyl methylglyoxal binds to $Na_V1.8$ sodium channels to reduce inactivation and increase the excitability of nociceptive neurons [3]. Extracellular application of MG also activates TRPA1 channels by reversible binding to cysteine residues following permeation of the cell membrane. MG-mediated activation of TRPA1 leads to a rise in the intracellular

[1]Institute of Pharmacology, Heidelberg University, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany [2]Vth Department of Medicine, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany [3]Department of Medicine I and Clinical Chemistry, University Hospital Heidelberg, Germany [4]German Center for Diabetes Research (DZD), Germany [5]Institute for Diabetes and Cancer IDC Helmholtz Center Munich, Neuherberg, Germany [6]Joint Heidelberg-IDC Translational Diabetes Program, Dept. of Medicine I, Heidelberg University Hospital, Heidelberg, Germany [7]Neurobiology Laboratory, National Institute of Environmental Health Sciences, North Carolina, USA [8]Institute for Biomedical Research (BIOMED), School of Medical sciences, Catholic University of Argentina, Buenos Aires, Argentina

[9] Contributed equally.

*Corresponding author. Fax: +49 6221 54 8644. E-mail: marc.freichel@pharma.uni-heidelberg.de (M. Freichel).

Abbreviations: DR, Diabetic Retinopathy; GLO1, Glyoxalase 1; GSH, Glutathione; GSSG, Glutathione disulfide; HTA, Hemithioacetal; MEF, Mouse Embryonic Fibroblast; MG, Methylglyoxal; NO, Nitric oxide; RCS, Reactive carbonyl species; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; STZ, Streptozotocin; TRPC, Transient Receptor Potential Canonical

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calcium concentration and subsequent release of Calcitonin Gene-related Peptide (CGRP) [4]. TRPA1 mediates MG-evoked acute pain sensation [5], but the causal contribution of TRPA1 to long-term complications is not established. Other diabetes-associated reactive metabolites, such as ROS and RNS, also significantly modulate the activity of cation channels of the TRP family including TRPC channels [6,7].

TRPC channels (TRPC1-TRPC7) are mammalian homologs of *trp* channels, originally discovered in *Drosophila* photoreceptor cells. Structurally, these channels have six transmembrane domains, and the pore is formed by transmembrane domain 5 and 6. Based upon the structural homology, the TRPC family can be divided into three subgroups, TRPC1/TRPC4/TRPC5, TRPC3/TRPC6/TRPC7, and TRPC2. FRET based assays and immunoprecipitations, using synaptosomal protein fractions from cerebellum or cortex, have provided evidence for the interaction of TRPC1, TRPC4, and TRPC5, suggesting that they form heteromeric channels [8,9]. Recently, the formation of heteromeric channels by TRPC1, TRPC4, and TRPC5 was demonstrated using quantitative high-resolution mass spectrometry on affinity-purifications (APs) from total brain and in hippocampus neurons using isoform specific antibodies [10]. In the embryonic brain, TRPC1 and TRPC4 could also be co-immunoprecipitated with TRPC6 [11].The cation channels formed by TRPC proteins are permeant for Ca^{2+} and Na^{+} under physiological conditions and are activated in response to activation of phospholipase C-coupled receptors but also by various reactive metabolites accumulating under diabetic conditions [12,13]. For TRPC5, it has been shown that nitric oxide (NO) donors lead to S-nitrosylation of cysteine residues in the channel pore, evoking an increased open probability of the channel [14]. TRPC5 channel activity was also increased by application of the reduced form of thioredoxin [15] or by application of oxidised glutathione (GSSG) [16]. TRPC1 and TRPC4 modulate the sensitivity of TRPC5 channels towards RNS and ROS [14]. TRPC3, TRPC4, and TRPC6 were also found to be redox-sensitive channels, and their expression and activity were reported to be modulated by ROS species [17,18].

Numerous studies have investigated the differential regulation of members of the TRPC subfamily in experimental models of diabetes, including the streptozotocin (STZ) model, Zucker obese rat, Goto-Kakizaki rats, and *db/db* mice, as well as in human cells cultured under hyperglycemic conditions. However, depending on the study and the model system used, contradictory findings have been reported with respect to the transcription and expression of the TRPCs [19]. So far, the functional relevance of TRPC cation channels in diabetic complications was primarily analyzed at the cellular level, e.g. in cells contributing to microangiopathy and nephropathy such as cultured vascular smooth muscle cells, platelets or mesangial cells [19]. The relevance of TRPCs for diabetic complications has not been studied in complex disease models, in part, due to the lack of specific antagonists for individual TRPC channels, as well as the limitations of knock-down approaches for long-term studies in vivo.

To date, diabetic retinopathy is a prevalent complication, and is expected to increase in magnitude, given the global epidemic of type 2 diabetes, and the lack of a specific systemic treatment beyond glucose control. Treatments such as laser photocoagulation and intravitreal injections of anti-proliferative or anti-inflammatory agents aim at late disease stages, are invasive by nature, and have significant side effects. Diabetic retinopathy (DR) in rodents and humans affects almost all cell types and culminates in impaired function and structure from the point of inception. The initial vascular phenotype is vasoregression, i.e. the loss of pericytes and endothelial cells. Neurodegeneration leading to a reduction of retinal thickness can occur as a consequence of progressive retinal capillary drop-out [20] but also as an independent process involving progressive cell death by apoptosis. In this process, accumulation of reactive metabolites and MG can play an important role [20,21].

In this study, the causal contribution of four TRPC proteins, TRPC1, TRPC4, TRPC5, and TRPC6, was investigated with respect to their role in DR by comparing *Trpc1/4/5/6*$^{-/-}$(TRPC QKO) mice to wild-type controls in the STZ-induced model of diabetes.

2. METHODS

2.1. Mice

To analyze the mRNA expression level of TRPCs by Nanostring, we used wild-type and *Ins2*^{Akita} mice with C57BL/6J background as a model for type 1 diabetes [22]. The quadruple Trpc knockout mouse line *Trpc1/4/5/6*$^{-/-}$ was generated by intercrossing mice of the four mouse lines *Trpc1*$^{-/-}$ [23], *Trpc4*$^{-/-}$ [24], *Trpc5*$^{-/-}$ [25], and *Trpc6*$^{-/-}$ [26] mice. The *Trpc1/4/5/6*$^{-/-}$ mice had a mixed C57Bl6-129SvJ genetic background, and age and sex matched first generation (F1) offspring of C57Bl6/N and 129SvJ matings were used as controls. *Trpc1/4/5/6*$^{-/-}$ mice were routinely genotyped using primers specific for the corresponding Trpc-deficient alleles as described before [23–26]. Both mouse lines were bred and maintained at our university's Specific Pathogen Free (SPF) central animal facility. We used 8–12 weeks old male mice and treated them with STZ (Sigma–Aldrich, Taufkirchen, Germany) as described previously [3]. Briefly, mice received one STZ injection/day i.p. (60 mg/kg) for five days, and blood glucose levels were maintained in the range of 300–500 mg/dl by insulin glargine treatment twice weekly according to blood glucose levels (Accu-Chek Aviva, Roche, Mannheim, Germany). Glycated hemoglobin (HbA_{1c}) was determined by cation-exchange chromatography on a PolyCAT A column [27]. Mice were kept in standard 12 h light/dark cycle and provided free access to standard chow diet and water. Markers for retinopathy, neurodegeneration, and methylglyoxal measurement were analyzed 30 weeks after the development of hyperglycemia. All animal experiments were conducted in accordance with the relevant guidelines by the EU Directive 2010/63/EU and approved by the local Animal Care and Use Committee at the regional authority in Karlsruhe, Germany.

2.2. Analysis of TRPC expression in retina

Total RNA was extracted from retinae of 8-month-old *Ins2*^{Akita} mice and nondiabetic control mice using TRIZOL method (Thermo Fisher, Germany). RNA concentrations were measured using spectrophotometer (Infinite 200 PRO Nanoquant, TECAN, Austria) and microfluidic analysis (Bioanalyzer 2100, Agilent Technologies, USA). Analysis of the transcripts was done by NCounter Nanostring technology in a three-step method described by Geiss et al. [28]. Briefly, in the first step, two probes, the reporter and the capture probe, hybridize directly to the target molecule in solution. Then, the target–probe complexes are immobilized on the imaging surface of the nCounter Cartridge by binding to the capture probe. Finally, the sample cartridges are scanned by an automated fluorescence microscope, and molecular barcodes (fluorophores contained in the reporter probe) for each specific target are counted. For expression analysis by NCounter NanoString technology, 1 μg total RNA was hybridized (four biological replicates, RIN$>$ 8.3) with a Nanostring Gene Expression CodeSet and analyzed using the nCounter Digital Analyzer (Nanostring Technologies, Seattle, USA). Background correction was performed, and normalization was applied using 5 different reference genes (Hprt1, Tbp, Ubc, Gapdh, Actb). Trpc specific DNA sequences used are listed in Supplementary Table 1.

