High glucose-induced hyperosmolarity contributes to COX-2 expression and angiogenesis: implications for diabetic retinopathy

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Abstract

Background: We tested the hypothesis that glucose-induced hyperosmolarity, occurring in diabetic hyperglycemia, promotes retinal angiogenesis, and that interference with osmolarity signaling ameliorates excessive angiogenesis and retinopathy in vitro and in vivo.

Methods and Results: We incubated human aortic (HAECs) and dermal microvascular endothelial cells (HMVECs) with glucose or mannitol for 24 h and tested them for protein levels and in vitro angiogenesis. We used the Ins2 Akita mice as a model of type 1 diabetes to test the in vivo relevance of in vitro observations. Compared to incubations with normal (5 mmol/L) glucose concentrations, cells exposed to both high glucose and high mannitol (at 30.5 or 50.5 mmol/L) increased expression of the water channel aquaporin-1 (AQP1) and cyclooxygenase (COX)-2. This was preceded by increased activity of the osmolarity-sensitive transcription factor Tonicity enhancer binding protein (TonEBP), and enhanced endothelial migration and tubulization in Matrigel, reverted by treatment with AQP1 and TonEBP siRNA. Retinas of Ins2 Akita mice showed increased levels of AQP1 and COX-2, as well as angiogenesis, all reverted by AQP1 siRNA intravitreal injections.

Conclusions: Glucose-related hyperosmolarity seems to be able to promote angiogenesis and retinopathy through activation of TonEBP and possibly increasing expression of AQP1 and COX-2. Osmolarity signaling may be a target for therapy.

Keywords: Glucose, Diabetes, Hyperosmolarity, Cyclooxygenase-2, Angiogenesis, Microangiopathy, Diabetic retinopathy

Background

Hyperglycemia can cause diabetic micro- [1] and macrovascular complications [2] by triggering oxidative stress [3, 4], forming advanced glycation end products (AGEs) [4, 5], increasing the flux of glucose to sorbitol through the polyol and hexosamine pathways [3], activating protein kinase C (PKC) [6], and provoking inflammation [7] (reviewed in [8]). Diabetic microvascular disease is pathologically characterized by excessive vessel growth and increased vessel permeability [9, 10].

Hyperglycemia has been reported to increase the endothelial expression of the proinflammatory proteins intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, and to decrease production of nitric oxide, all through activation of the water channel protein aquaporin (AQP)1 [11]. Hyperosmotic stress inevitably accompanies hyperglycemia, and may
therefore contribute to some aspects of hyperglycemia-induced vascular injury.

Similarly to VCAM-1 and ICAM-1, cyclooxygenase (COX)-2 participates in inflammation [12] and angiogenesis [13, 14]. Induced by cytokines, mitogens and endotoxin [12], COX-2 regulates the expression and activity of matrix metalloproteinase (MMP)-9 [15], which degrades extracellular matrix and promotes endothelial cell migration and vessel sprouting [16]. Endothelial COX-2 has been implicated in angiogenesis (mostly in the context of tumorigenesis, reviewed in [17]), as well as in proliferative diabetic retinopathy [18], characterized by excessive angiogenesis [19–21]. COX-2 is expressed in human monocytes and macrophages, as well as human endothelial cells [22] exposed to high glucose [7].

Against this background, we therefore examined whether hyperosmotic stress, at levels attainable in diabetes, induces COX-2 expression in human micro- and macrovascular endothelial cells. We then analyzed the functional impact of hyperosmotic stress and COX-2 expression on angiogenesis and explored underlying mechanisms, particularly with regard to the involvement of water channels and associated signaling molecules. Finally, we investigated the in vivo implications of such findings for diabetic retinopathy in a mouse model.

**Methods**

**Materials**

D-glucose, D-mannitol and l-glucose (these two latter devoid of metabolic activities and used as a purely hyperosmolar controls), saccharose, and sodium chloride, were purchased from Sigma (St. Louis, MO, USA). The selective COX-2 inhibitor NS-398 was purchased from Calbiochem (Gibbstown, NJ, USA).

**Animal care**

Male C57BL/6 mice (body weight: 15±4 g, age: 1 year; n=21) and male D2.B6-Ins2 Akita diabetic mice, a model of type 1 diabetes developing retinal angiogenesis [23, 24] (body weight: 11±2 g, n=21) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). All procedures were approved by our Institutional Ethics Committee for Animal Research. Investigations conformed to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals [NIH Publication 86–23, 1985 revision].

**Cell cultures**

Subconfluent human aortic endothelial cells (HAECs) and human microvascular endothelial cells (HMVECs) at passage 3 were synchronized by starvation in Endothelial Basal Medium (EBM, Clonetics, Baltimore, MD, USA) for 24 h, then incubated with control d-glucose concentration (5.5 mmol/L, control and 285 mOsm/L), high glucose (HG: 30.5 mmol/L and 385 mOsm/L or 50.5 mmol/L and 460 mOsm/L), high mannitol (HM: 5.5 mmol/L glucose + 25 mmol/L and 385 mOsm/L or 45 mmol/L mannitol and 385 mOsm/L), for 24 or 72 h. In some experiments, cells were also incubated with l-glucose at equimolar concentrations, saccharose or sodium chloride, as further osmotic controls, in the presence or absence of 1–10 μg/mL NS-398.

Cell viability after treatments was assessed by cell morphology and cell count at phase-contrast microscopy, Trypan blue exclusion, and total protein content.

**Immunoblotting**

Total proteins from retinas of wild-type C57BL/6 male mice and sex/age matched D2.B6-Ins2 Akita diabetic mice, or from HMVECs and HAECs were isolated, electroblotted, and incubated with the following primary and secondary antibodies: (1) goat polyclonal anti-COX-2 (cat. N° sc-1747, dilution 1:500, Santa Cruz Biotechnologies, Santa Cruz, CA USA), secondary antibody mouse anti-goat IgG-HRP (cat. N° sc-2354, dilution 1:5000, Santa Cruz); (2) mouse monoclonal anti-AQP1 (cat. N° sc-55466, dilution 1:600, Santa Cruz), secondary antibody goat anti-mouse IgG-HRP (cat. N° sc-2005, dilution 1:5000, Santa Cruz); (3) mouse monoclonal anti-β-actin (cat. N° A5441, dilution 1:5000, Sigma, St. Louis, MO, USA), secondary antibody goat anti-mouse IgG-HRP (cat. N° sc-2005, dilution 1:5000, Santa Cruz); (4) rabbit polyclonal anti-TonEBP (cat. N° sc-13035, dilution 1:500, Santa Cruz), secondary antibody goat anti-rabbit IgG-HRP (cat. N° sc12304, dilution 1:10000, Santa Cruz), as previously described [25].

**Transfection of AQP1 and tonicity enhancer binding protein (TonEBP)/nuclear factor of activated T cells (NFAT)5 siRNA**

A pool of three different small interfering RNA (siRNA) oligonucleotides to each target (AQ1P1 and TonEBP/NFAT5), as well as scrambled control siRNA were incubated with cells (2×10^5/well) in transfection medium, as previously described [26]. After 24 h, fresh medium was added with or without HG (30.5 or 50.5 mmol/L), or HM (5.5 mmol/L glucose + 25 or 45 mmol/L mannitol), incubated for an additional 24 h, then subjected to Western analysis of COX-2, AQP1, TonEBP/NFAT5 and β-actin.

**Tube formation assays**

Tube formation assays were performed as previously described [26]. Before the assay, cells were incubated in low serum (2.5 %) endothelial basal medium (EBM, BD Biosciences) without endothelial growth factors, with concentrations of control glucose (5.5 mmol/L,
control), high glucose (30.5 or 50.5 mmol/L), high mannitol (5.5 mmol/L glucose + 25 or 45 mmol/L mannitol) for 24 h, in the presence or absence of the selective cyclooxygenase(COX)-2 inhibitor NS-398 at 1–10 μg/mL. In the assay testing the effect of siRNA to aquaporin (AQP1) or TonEBP, cells were pretreated with high glucose or high mannitol for 24 h. At the end of incubations, cells were gently detached with 0.25% trypsin–EDTA; trypsinized cells were then collected, counted and plated on Matrigel (at a density of 2 × 10^5 cells/50 μL Matrigel) for a further 16 h, in the presence or absence of control glucose, high glucose, high mannitol, with or without NS-398, or siRNA to AQP1 or TonEBP.

**Cell migration assay**

Cell migration was measured using the CytoSelect™ cell migration assay (Cell Biolabs, San Diego, CA, USA), according to the manufacturer’s instructions. Following a 24 h starvation period in 2% fetal calf serum (FCS)/endothelial cell growth medium (EGM), cells were resuspended at 4 × 10^5 cells/mL in EGM and then placed in a separate well of a 96-well feeder tray containing 150 μL of migration media (EGM with control glucose concentration (5.5 mmol/L), control), high glucose (HG 30.5 mmol/L) or high mannitol (HM HM, 5.5 mmol/L glucose + 25 mmol/L mannitol)). One hundred and fifty microliters of assay controls, consisting of EGM supplemented with 10% FCS (positive control) and basal EGM (negative control), were also tested in triplicate.

**Electrophoretic mobility gel shift assay**

Nuclear proteins were extracted from treated or untreated endothelial cells and subjected to electrophoretic mobility gel shift assay, as previously described [25]. Double-stranded synthetic oligonucleotides of the nuclear factor (NF)-κB motif (5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-CCT GGG AAA GTC CCC TCA ACT-3′) or of the Tonicity enhancer element (5′-GTT TTC TCC ACC TTT TCC GCT G-3′) were labeled with [γ-32P]ATP.

**Immunoblotting of TonEBP**

15 μg of nuclear extracts used for EMSA and of cytosolic extracts were electoblotted and incubated with either a primary rabbit polyclonal anti-TonEBP (cat. N° sc-13035, dilution 1:500, Santa Cruz), secondary antibody goat anti-rabbit IgG-HRP (cat. N° sc-12304, dilution 1:10,000, Santa Cruz); or an anti-lamin B antibody (Santa Cruz), as previously described [25].

**Preparation of transit TKO-siRNA complexes and intravitreal injections of non-viral siRNA**

Non-viral siRNA delivery into the mouse retina in vivo was done using a modified version of published methods [27]. For monitoring transfection efficacy we used scrambled siRNA conjugated with a Cy3 fluorochrome provided by Mirus (Mirus Bio, Madison, WI, USA). Accordingly, 1.33 μL (25 nM) of Cy3-conjugated scrambled siRNA or Cy3-conjugated AQP1-siRNA (Ambion) were combined with 0.5 μL of Transit-TKO (Mirus) in a final volume 10 μL sterile H2O, and incubated for 30 min at room temperature. Wild-type C57BL/6 mice and age-sex matched D2.B6-Ins2 Akita diabetic mice were anesthetized by intraperitoneal injection of ketanest/xylazine (ketanest, 100–125 mg/kg; xylazine, 10–12.5 mg/kg). Intravitreal injections of phosphate-buffered saline (PBS, sham group, N = 7 wild-type C57BL/6 mice and N = 7 D2.B6-Ins2 Akita mice), or scrambled-siRNA (N = 7 wild-type C57BL/6 mice and N = 7 D2.B6-Ins2 Akita mice), or AQP1-siRNA (N = 7 wild-type C57BL/6 mice and N = 7 D2.B6-Ins2 Akita mice) were performed under a dissecting microscope with a 32 gauge needle attached to a 5 μL glass syringe (Hamilton, Reno, NV, USA). The needle was positioned 1 mm posterior to the limbus, where 5 μL of the solution were slowly (in 3–5 s) injected into the vitreous chamber of the eye. Injections were repeated after 72 h and 1 week from the first injection.

**Tissue preparation and histological evaluation of vessel density**

After the induction of anesthesia, retinas from D2.B6-Ins2 Akita diabetic mice and age/sex-matched C57/BL6 control mice were removed, embedded in optimal-cutting-temperature (OCT) medium, frozen on dry ice, and stored at −70 °C until sectioning. For immunofluorescence staining, 5 μm thick sagittal and frontal cryosections were obtained by using a sliding cryotome. These sections were permeabilized, blocked for 30 min in PBS containing 1% bovine serum albumin, and incubated for 1 h at 4 °C with purified primary antibody anti-CD31 or Cy3-conjugated anti-α-smooth muscle actin (ASMA). A non-immune IgG (Becton, Dickinson and Co., Franklin Lakes, New Jersey, USA) was used as the isotype control. After washing with PBS, retinal sections stained with anti-CD31 antibody were incubated with phycoerythrin (PE)-conjugated secondary antibody, washed, mounted, and viewed with an immunofluorescence microscope. Sections were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) to identify nuclei. For immunohistochemistry, retinas were embedded in five separate paraffin blocks. Paraffin-embedded limb sections were deparaffinized, rehydrated, and stained with a primary antibody against CD31. Sections were counterstained with hematoxylin and eosin for structural analysis and to identify nuclei. Arterioles and capillaries were counted in a blinded manner in five randomly selected fields at 10× magnification. Vascular images were captured by using...
an inverted light microscope (Olympus IX71), and analyzed by using the Image-J software (Media Cybernetics, Rockville, MD, USA). Vessel density was expressed as the number of arterioles or capillaries per mm².

Statistical analysis
Two-group comparisons were performed by the Student’s t test for unpaired values. Comparisons of means of ≥3 groups were performed by analysis of variance (ANOVA), and the existence of individual differences, in case of significant F-values at ANOVA, tested by Scheffe’s multiple contrasts.

Results
High glucose induces COX-2 expression in human endothelial cells through a hyperosmolar mechanism
Both short-term and longer-term exposure to high concentrations of glucose or mannitol, increased the expression of COX-2 protein, as shown by Western analysis (Fig. 1a–g). Similar results were obtained by incubating HAECs and HMVECs with l-glucose and NaCl at equimolar concentrations (30.5 mmol/L), as a further osmotic controls (Fig. 1h–i).

AQP1 mediates high glucose-induced COX-2 expression in endothelial cells
Since AQP1 regulates responses to hyperosmolarity in endothelial cells [28], we tested whether AQP1 is involved in hyperosmolarity-induced COX-2 expression. We used targeted disruption of AQP1 gene expression by siRNA technology, with 3 different siRNA species, to specifically block AQP1. AQP1 siRNA did not affect cell morphology or cell proliferation (Fig. 2a), but triggered a substantial (80 %) reduction in AQP1 protein expression (Fig. 2b). By contrast, serving as a sequence-unrelated control, scrambled siRNA did not exert any detectable effect. TonEBP/NFAT5 silencing drastically decreased the network number and area formed by HAECs seeded in Matrigel, compared with those obtained from glucose and mannitol-treated cells (Fig. 3E). Cell migration induced by high glucose or high mannitol in a modified Boyden chamber system was also quantified with a fluorimetric assay. Migration of TonEBP/NFAT5-deficient endothelial cells towards high glucose and high mannitol was significantly inhibited (Fig. 3F), indicating a role of TonEBP/NFAT5 in mediating hyperosmolarity-induced endothelial migration and angiogenesis. TonEBP/NFAT5 expression and binding activity was increased in cells exposed to high glucose and mannitol (Fig. 4a–d), and siRNA against TonEBP/NFAT5 24 h prevented the hypertonic induction of TonEBP/NFAT5 (Fig. 4a) and COX-2 proteins (Fig. 5a), indicating that TonEBP/NFAT5 expression and activity are required for the hyperosmolarity induction of COX-2. Taken together, these results showed that hyperosmolarity contributes to the stimulatory effect of COX-2 on endothelial angiogenesis mostly through the activation of upstream and downstream mediators of hyperosmolarity, such as AQP1 and TonEBP/NFAT5, respectively.

The promoter region of the COX-2 gene also contains consensus sequences for NF-κB [33–36], and high glucose induces the expression of some genes also by activating NF-κB [33, 37]. In order to analyze the contribution of NF-κB to the hyperosmotic stress-induced expression of COX-2, we performed EMSA using NF-κB binding probe and nuclear extracts from osmotically stressed HAECs for 1–24 h. We found low, but detectable amounts of NF-κB DNA binding activities in unstimulated cells. After treatment with high glucose (1 and 3 h) and TNF-α (up to 24 h), but not with high mannitol or sodium chloride, we observed a time-dependent increase in NF-κB DNA-binding activity, suggesting that this transcription factor can cooperate to the expression of the
COX-2 gene by high glucose, but not in response to high glucose-related hyperosmotic stress (Fig. 5b–c).

Retinas of D2.B6-Ins2 Akita diabetic mice exhibit high levels of hyperosmolarity-enhanced COX-2 expression and angiogenesis

CD31 (a marker of angiogenesis) and α-smooth muscle actin (ASMA, a marker of arteriogenesis), along with COX-2 and AQP1 were examined in retinas of D2.B6-Ins2 Akita diabetic mice and sex-age matched C57BL/6 non-diabetic controls treated with or without siRNAs to AQP1. We detected low-level CD31 (Fig. 6a) and ASMA immunoreactivities (Fig. 7a–d) in WT mice. However, a significant vessel staining for CD31 (Fig. 6b) and ASMA (Fig. 7g–l) were apparent in retinas of D2.B6-Ins2 Akita mice, mostly in the inner plexiform layer (IPL) and Outer plexiform layer (OPL). Retinas in diabetic mice also showed increased expression of AQP1 and a significant upregulation of COX-2 at the protein level (Fig. 6d–e). AQP1 in vivo silencing drastically decreased the number

**Fig. 1** The effect of high glucose and high mannitol on COX-2 expression in endothelial cells. Immunoblotting with an antibody against COX-2 of proteins extracted from serum-starved subconfluent human aortic endothelial cells (HAECs) incubated with control D-glucose (5.5 mmol/L, control), high glucose (HG, 30.5 or 50.5 mmol/L), high mannitol (HM, 5.5 mmol/L glucose + 25 or 45 mmol/L mannitol) for 24 h (a, b) and for 3 days (c, d). After COX-2 analyses, proteins were stripped and restained with an anti-β-actin antibody. Lipopolysaccharide (LPS, 1 μg/mL) was used as positive control for COX-2 expression (a, d). e, f Immunoblotting with an antibody against COX-2 of proteins extracted from serum-starved subconfluent human microvascular endothelial cells (HMVECs) incubated with stimuli (12.5 and 25 mmol/L glucose or mannitol) for 3 days. Representative immunoblots are shown. The results of scanning densitometry (n = 3 independent experiments) are expressed as arbitrary units in panel g. Columns and bars represent the mean ± SD (*, P < 0.05 vs control cells; **, P < 0.01 vs control cells). Equal loading/equal protein transfers were verified by protein band detection with the anti-β-actin antibody. h, i The effect of high D-glucose, high l-glucose, high mannitol and NaCl on COX-2 expression in endothelial cells. Immunoblotting with an antibody against COX-2 of proteins extracted from serum-starved subconfluent human aortic endothelial cells (HAECs) incubated with control glucose concentration (5.5 mmol/L, control), high glucose (30.5 mmol/L), high mannitol (5.5 mmol/L glucose + 25 mmol/L mannitol), or sodium chloride (30.5 mmol/L), for 24 h. After COX-2 analyses, proteins were stripped and restained with an anti-β-actin antibody. Representative immunoblots from three different experiments are shown. i Columns and bars represent the mean ± SD of scanning densitometry of panel H (*, P < 0.05 vs control cells; **, P < 0.01 vs control cells).
of ASMA-positive vascular structures (Fig. 7e–f, m–n) and the expression of AQP1 and COX-2 (Fig. 8a, b), in D2.B6-Ins2 Akita mice.

Discussion
Clinical consequences of type-1 diabetes result from the combination of a number of different factors, ranging from acidosis to metabolic changes, to bio-physical derangements, such as hyperosmotic stress. Previous research has mostly focused on metabolic disorders induced by hyperglycemia and the underlying signaling pathways. Several lines of evidence presented here however indicate that a substantial contribution to the induction of vascular disease is given by the hyperosmolar stress related to hyperglycemia. We show here that concentrations of glucose attainable in diabetes induce COX-2 in human endothelial cells through an AQP1-dependent hyperosmolar mechanism and, through this mechanism, promote angiogenesis. Specifically, in diabetic Ins2Akita mice, retinal COX-2 and AQP1 are upregulated concomitant with increased angiogenesis, suggesting the relevance of our observations in an in vivo setting where hyperglycemia is the main driver of microvascular disease. Finally, hyperosmolarity, through an AQP1-dependent mechanism appears to be causally involved in angiogenesis in vivo, and interruption of the AQP1 axis mitigates in vivo increased angiogenesis in response to high glucose.
This report expands our previous findings that hyperosmotic stress induces endothelial ICAM-1 and VCAM-1 expression [11] by showing the involvement of COX-2-dependent pathological phenomena. The current findings provide evidence that hyperosmolarity is a biophysical mechanism through which excessive angiogenesis may occur in diabetes. Additionally, our findings detail part of the molecular signaling through which hyperosmolarity promotes such effects.

The involvement of AQP1 in hyperosmolarity-induced endothelial tubulization and angiogenesis [38–41] is of biological importance. AQP1 is a water channel protein widely expressed in vascular endothelial cells, which, when activated, increases cell membrane water...
permeability [42–44]. AQP1 protein is strongly expressed in human and animal models of highly proliferating microvessels [43] and the chick embryo chorioallantoic membrane [44]. In human retinal epithelial cells, osmotic stress decreases AQP4 expression [45]. Reduction in AQP1 expression by targeted siRNA inhibits hyperosmolarity-dependent angiogenesis. In general, angiogenesis requires endothelial cell proliferation, adhesion and migration. We found a significant decrease in network number and areas in AQP1-silenced cultures, indicating a lost capacity to organize tubuli in networks. This is in line with previous reports showing that AQP1 plays a key role in endothelial cell migration, an early fundamental step in angiogenesis [10, 46].

Data from the present study also provide a mechanistic explanation on how AQP1 is linked to endothelial cell
migration and tubulization. We observed that high glucose, at levels of 30.5 mmol/L, corresponding to 450 mg/dL, a plasma level possibly found in uncontrolled diabetes—increases COX-2 expression. This effect is in part dependent on the hyperosmolar component of hyperglycemia, since mimicked by the exposure of cells to the metabolically inactive monosaccharides mannitol or l-glucose.

TonEBP/NFAT5, a rel/NF-kB family member, has been identified as a transcription factor that in response to hyperosmolarity activates the transcription of osmosensing genes, including aldose reductase and protein kinase C delta [47], TNF-α and monocyte chemoattractant protein (MCP)-1 [30, 34–36]. In renal medullary epithelial cells, hypertonic stress increased COX-2 promoter activity in a luciferase reporter assay [37, 48].

**Fig. 5** The involvement of TonEBP/NFAT5 in glucose-induced endothelial COX-2 expression. 

a Western analysis of the effect of siRNA to TonEBP/NFAT5 on COX-2 expression in endothelial cells exposed to high glucose and high mannitol. Serum-starved human aortic endothelial cells (HAECs) were transfected with fluorescein isothiocyanate (FITC)-conjugated-siRNA to TonEBP/NFAT5 or siRNA to scrambled sequence for 24 h, and then treated with stimuli for 24 h with high glucose, or high mannitol, or equimolar concentration of sodium chloride. At the end of treatments transfection efficiency was checked by observing the plates under fluorescence microscopy. Protein expressions for cyclooxygenase (COX)-2 was detected by Western analysis, with β-actin serving as loading control. Data represent for each condition the mean ± S.D. from three separate experiments. **, P < 0.01 mannitol- or glucose-treated vs control HAECs; §, P < 0.05 vs without TonEBP/NFAT-siRNA.

b The effect of hyperosmotic stress on NF-kB activation in human aortic endothelial cells. Electrophoretic mobility gel shift assay (EMSA), showing the effect of hypertonic stress on NF-kB activation. EMSA was performed by mixing the 32P-oligonucleotide encoding for the NF-kB binding probe with nuclear extracts from osmotically stressed HAECs for 1–3 h. The supershift analysis with a polyclonal rabbit antibody against p65, shows upward shift of binding complexes. Results are representative of three separate experiments.

c Scanning densitometry of the shift bands (highlighted within the rectangle) of EMSA gels, expressed as arbitrary units of optical density. Data represent for each condition the mean ± S.D. from 3 separate experiments. *, P < 0.01 vs control HAECs
Also, hypertonic saline had been reported to induce COX-2 in rat macrophages [49] and HUVECs [50], but in experimental conditions that do not reproduce the diabetic milieu. The participation of NFAT transcription factors in the activation of COX-2 expression had also been already demonstrated in several cell types [31, 32, 51–54]. Indeed, previous research had shown that the angiogenic signaling involved in the induction of COX-2 by vascular endothelial growth factor (VEGF) in human endothelial cells requires the activation of NFAT proteins [51, 55]. Furthermore, TonEBP/NFAT5 is required for the hypertonic induction of COX-2 in renal epithelial cells [31]. An upregulation of the NFAT-MMP-2 pathway had been also demonstrated in the development of metastatic osteosarcoma, where the secretion of active MMP-2 is under the transcriptional regulation of NFAT [32]. Here however we provide the first evidence that the induction of COX-2 in endothelial cells is largely hyperosmolarity-related, and that TonEBP/NFAT5 is here involved, since (a) TonEBP/NFAT total abundance was increased in cells exposed to high glucose or high mannitol; (b) EMSA and Western analysis demonstrated a cytoplasm-to-nucleus redistribution of TonEBP/NFAT in endothelial cells; and (c) TonEBP/NFAT gene disruption prevented hyperosmolarity-related induction of COX-2. Of note, our data show that at least another signal transduction pathways

![Fig. 6 Retinal neovascularization and higher expression of AQP1 and COX-2 in the Ins2Akita mice.](image)
Fig. 7  The involvement of AQP1 in retinal neovascularization in the Ins2Akita and C57BL/6 mice. Immunofluorescence staining of retinal cross-sections for α-smooth muscle actin (ASMA) and 4′,6-diamidino-2-phenylindole (DAPI) for nuclei from D2.B6-Ins2 Akita (G-N) and sex- and age-matched (1 year-old) C57BL/6 non-diabetic control mice (a–f), after intravitreal injection of saline (sham, panels a, b, g, h), or siRNA with scrambled sequence (siRNA-scr, panels c, d, i, l) or siRNA against AQP1 (siRNA-AQP1, panels e, f, n, m). Magnification: 10x

Fig. 8  The involvement of AQP1 in COX-2 expression in the Ins2Akita and C57BL/6 mice. a Western analysis of COX-2 and AQP1 expression in retinas from from D2.B6-Ins2 Akita and sex- and age-matched (1 year-old) C57BL/6 non-diabetic control mice, after the intravitreal injection of saline (sham), or siRNA against scrambled sequence (siRNA-scr) or siRNA against AQP1 (siRNA-AQP1), with β-actin serving as a loading control. b n = 2 eyes per animal, with each experimental group consisting of n = 7 mice. The bar graph represents the means ± S.D. from three separate experiments. **, P < 0.01 vs C57BL/6 control mice ††, P < 0.01 vs sham and siRNA-scr. Glyco AQP1 represents the glycosylated form of AQP1 able to insert itself in the plasma membrane.
activated by high glucose, but with lesser relevance to COX-2 expression and angiogenesis, such as the NF-κB pathway, is—conversely—not substantially induced by high mannitol, indicating a larger dependence of NF-κB activation on metabolic effects of glucose. A number of studies have shown that hyperosmolarity (as tested by high mannitol) does not influence angiogenesis and/or COX-2 expression in endothelial cells [55, 56]. Culture conditions used in these studies may not reflect those used in our study. Our data show more robust effects when using glucose concentrations as high as 50.5 mM, or with lower concentrations but for prolonged times, which may more closely mimic diabetic pathophysiology. Concentrations of glucose outside the range tested here might exert different effects, therefore not completely ruling out the additional involvement of mechanisms different from hyperosmolarity. Nevertheless, our data clearly indicate that hyperosmolarity is an important part of the mechanisms through which chronic exposure to high glucose may promote angiogenesis and cardiovascular complications of diabetes.

We recognize some limitations of our study. Main ones are that (a) there is limited translatability of murine data to humans; (b) molecular mechanisms by which TonEBP/NFAT operates on the COX-2 promoter are still incompletely understood; (c) we still need to close the circle of the in vivo causality demonstration involving the transcription factor TonEBP/NFAT5 as an additional osmosignaling effector by showing that the in vivo interruption of the TonEBP signaling mitigates the in vivo increased angiogenesis in response to high glucose. Such experiments are complicated by the lack of available transgenic mouse models of TonEBP knockouts. Such limitations might however be overcome in the future through the prolonged administration of drugs targeting TonEBP activation.

Nonetheless, data provided here lend support to the hypothesis that hyperosmolarity is an important mechanism of vascular disease in diabetes and a potential target for future therapies, with several potential practical implications. In principle, promotion of inflammation and angiogenesis by COX-2 through a hyperosmolarity-related mechanism may have a role not only in microvascular disease (proliferative retinopathy), as shown here, but also in macrovascular disease (e.g., plaque rupture), both increased in diabetes. The unraveling of the hyperosmolarity-related signaling, as sketched in Fig. 9, pointing both to the sensing molecule AQP1 and the effector molecule COX-2, allows identifying potential pharmacological targets to reduce hyperosmolar stress-related excessive angiogenesis. In addition, the in vivo expressions of AQP1 and COX-2 might serve as new markers for diabetic complications. The in vivo implications of these observations are therefore worth further assessment.

**Authors’ contributions**
RM designed and performed the experiments, analyzed the data and wrote the manuscript; GG performed in vitro experiments and analyzed the data; PC performed in vitro experiments; FVR performed in vitro experiments; YJG conceived the experiments, provided advise and financial support, and corrected the final manuscript. RDC conceived, designed and interpreted the experiments, and corrected the final manuscript. All authors read and approved the final manuscript.

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**Competing interests**
The authors declare that they have no competing interests.

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