TRPM7 channels play a role in high glucose–induced endoplasmic reticulum stress and neuronal cell apoptosis

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High-glucose (HG) levels and hyperglycemia associated with diabetes are known to cause neuronal damage. The detailed molecular mechanisms, however, remain to be elucidated. Here, we investigated the role of transient receptor potential melastatin 7 (TRPM7) channels in HG-mediated endoplasmic reticulum stress (ERS) and injury of NS20Y neuronal cells. The cells were incubated in the absence or presence of HG for 48 h. We found that mRNA and protein levels of TRPM7 and of ERS-associated proteins, such as C/EBP homologous protein (CHOP), 78-kDa glucose-regulated protein (GRP78), and inducible nitric-oxide synthase (iNOS), increased in HG-treated cells, along with significantly increased TRPM7-associated currents in these cells. Similar results were obtained in cerebral cortical tissue from an insulin-deficiency model of diabetic mice. Moreover, HG treatment of cells activated ERS-associated proapoptotic caspase activity and induced cellular injury. Interestingly, a NOS inhibitor, L-NAME, suppressed the HG-induced increase of TRPM7 expression and cellular injury. siRNA-mediated TRPM7 knockdown or chemical inhibition of TRPM7 activity also suppressed HG-induced ERS and decreased cleaved caspase-12/caspase-3 levels and cell injury. Of note, TRPM7 overexpression increased ERS and cell injury independently of its kinase activity. Taken together, our findings suggest that TRPM7 channel activities play a key role in HG-associated ERS and cytotoxicity through an apoptosis-inducing signaling cascade involving HG, iNOS, TRPM7, ERS proteins, and caspases.

Diabetes mellitus is one of the most serious metabolic disorders in humans with encephalopathy as a common complication. Hyperglycemia has been considered the main pathogenic factor underlying the development of diabetic complications, although other factors have also been implicated (1–4). The exposure of neurons to high concentrations of glucose (HG), along with microvascular injury, is considered a major cause of diabetic neuropathy (5–8). Neuronal damage caused by HG or hyperglycemia is known as glucose neurotoxicity (7). The detailed molecular mechanisms underlying HG-induced neuronal injury, however, remain to be elucidated.

Apoptotic cell death is common in diabetes and neurodegenerative disorders affecting the central nervous system (9, 10). For example, apoptotic neuronal loss has been shown in the hippocampus and cerebral cortex of type 1 and type 2 diabetic patients and rodents (11, 12). Three apoptotic signaling pathways have been discovered: 1) the mitochondrial pathway; 2) the death receptor-associated pathway; and 3) the endoplasmic reticulum (ER)-associated pathway (14–16). Recent studies indicated that ER stress (ERS)-mediated apoptosis participates in HG-induced cell death (10, 13, 14).

ER plays a pivotal role in various cellular processes, including synthesizing, sorting, assembling, trafficking of proteins, and maintaining intracellular Ca2+ homeostasis (15). Accumulation of misfolded or unfolded proteins in the ER, caused by impairment of Ca2+ homeostasis and other harmful stimuli, triggers ERS response (16, 17). At the initial stage of this response, at least three resident ER transmembrane proteins that act as sensors, including PERK, ATF6, and IRE1, are activated. In the resting cells, these sensors remain in an inactive state through their association with chaperones, such as GRP78. Accumulation of unfolded proteins leads to their dissociation from GRP78 and activation of these sensors, triggering unfolded protein response (UPR) (18, 19). A short-term

3 The abbreviations used are: HG, high-glucose; L-NAME, Nω-nitro-L-arginine methyl ester; ER, endoplasmic reticulum; ERS, ER stress; UPR, unfolded protein response; CHOP, C/EBP homologous protein; TRP, transient receptor potential; NOS, nitric-oxide synthase; INOS, inducible nitric-oxide synthase; nNOS, neuronal NOS; pIC, picofarads; LG, L-glucose; DG, D-glucose; 2-APB, 2-aminoethoxydiphenyl borate; RIPA, radioimmune precipitation assay; ROS, reactive oxygen species; FBS, fetal bovine serum; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
**TRPM7 in hyperglycemia-mediated ER stress**

existence of UPR acts as a prosurvival response by reducing deposition of unfolded proteins and restoring normal ER function. However, if UPR persists, it will cause a series of complex network responses and activation of pro-apoptotic signal pathways through CCAAT/enhancer-binding protein homologous protein (CHOP) and caspase-12, ultimately leading to apoptotic cell death (20, 21).

TRPM7 is a unique member of the transient receptor potential (TRP) channel superfamily. It can be activated by oxidative stress and is permeable to Ca^{2+}, Zn^{2+}, and Mg^{2+} (22–25). Increasing evidence suggests that activation of TRPM7 channels contributes to various physiological and pathophysiological processes, including Mg^{2+} and Ca^{2+} homeostasis (24–26), tumor cell growth/proliferation (27–29), and cardiovascular functions (23, 30–32). We and others also found that these channels play an important role in normal neuronal functions, such as neurite outgrowth and vesicular transport, and in neuronal injury associated with neurological disorders, such as brain ischemia (22, 33–35). Recent studies have shown that the activity and/or expression of TRPM7 channels in nonneuronal cells (e.g. immune and vascular cells) are increased under HG conditions (31, 36), suggesting a role for these channels in the pathophysiology of diabetes. However, it has not been demonstrated whether TRPM7 channels play a role in ERS and HG-mediated neurotoxicity.

Here, we provide strong evidence showing that increased expression and activity of TRPM7 channels is associated with HG-induced ERS and resultant injury of neuronal cells. We further show that the level of inducible NO synthase (iNOS) is increased and that inhibition of NOS decreases the level of TRPM7 and cell injury. Together, our data suggest that up-regulation of TRPM7 channels by oxidative stress probably contributes to HG-mediated ERS and pro-apoptotic activity in neuronal cells.

**Results**

**Increased expression of Trpm7, Inos, Chop, and Grp78 mRNA in brain tissues isolated from an insulin-deficiency model of diabetic mice**

Previous studies of our own and others have shown that the level of TRPM7 expression is increased in nonneuronal cells under HG or diabetic conditions (31, 36). However, the effect of HG or diabetes on the level of TRPM7 in brain tissues or neuronal cells remains unknown. For this reason, we first examined the potential change of TRPM7 expression in brain tissues isolated from an insulin-deficiency model of diabetic mice. Diabetic mice were created by injecting alloxan (37) and confirmed by blood glucose level, as detailed under “Experimental procedures.” Cerebral cortices were collected 2 weeks after alloxan injection, followed by quantitative PCR. As shown in Fig. 1A, compared with the control group, the relative level of Trpm7 mRNA increased in the cortex from diabetic mice (1.31 ± 0.27, p < 0.01; n = 8–11 mice). Because diabetic condition facilitates oxidative stress and ERS (38, 39), we also examined the changes in the levels of Nos, Chop, and Grp78. We found that the levels of Inos, Chop, and Grp78 mRNA were augmented in brain tissues from the diabetic mice, whereas Nnos was slightly decreased (Inos, 1.55 ± 0.57; Grp78, 1.83 ± 0.66; Chop, 1.65 ± 0.48; Nnos, 0.49 ± 0.28; *, p < 0.05; **, p < 0.01; n = 8–11 mice; Fig. 1A). We also performed Western blotting to examine the potential change of TRPM7 and CHOP at the protein level. We saw a clear increase of both proteins in the cerebral cortex of an insulin-deficiency model of diabetic mice (TRPM7, 1.53 ± 0.16 (**, p < 0.01); CHOP, 1.79 ± 0.63 (*, p < 0.05); n = 5–6 mice; Fig. 1B).

**HG increases expression of TRPM7, iNOS, and ERS-associated molecules in NS20Y cells**

To better understand the relationship between TRPM7 and ERS-associated molecules in hyperglycemia-mediated neuronal cell damage, we performed in vitro studies using a mouse neuronal cell line, NS20Y cells (40), and determined the effect of hyperglycemia on the level of these molecules. Because of their high metabolic requirements, culture medium containing 25 mM glucose (control conditions) is essential for neurons and neuronal cell lines. It has been reported that increasing the glucose concentration above 35 mM induces hyperglycemic stress, ROS production, and injury of these cells (6). In the current study, 20 mM additional glucose was added to the normal culture medium to mimic the hyperglycemic condition. Compared with cells cultured under control condition (25 mM D-glucose), the relative levels of Trpm7, Inos, Grp78, and Chop mRNA in HG cultured cells (48 h) were significantly increased to 1.26 ± 0.03, 1.98 ± 0.55, 1.43 ± 0.40, and 1.57 ± 0.25, respectively (*, p < 0.05; **, p < 0.01; n = 3–4; Fig. 2A). These results
are consistent with those obtained from diabetic mouse brain (shown in Fig. 1). An additional experiment comparing NS20Y cells cultured in medium containing 10 mM glucose with those cultured in medium containing 25 mM glucose also showed a clear increase of TRPM7 and GRP78 expression (*, p < 0.05; n = 6).

We further examined the potential change of TRPM7, CHOP, GRP78, and iNOS protein under HG conditions. As shown in Fig. 2A, treatment of NS20Y cells with HG for 48 or 72 h significantly increased the relative level of TRPM7 protein expression to 1.58 ± 0.28 and 1.83 ± 0.47 (*, p < 0.05; n = 6).

CHOP, GRP78, and iNOS protein expression were also increased by HG treatment measured at 48 h (CHOP, 1.43 ± 0.29; GRP78, 1.45 ± 0.37; iNOS, 1.66 ± 0.21; Fig. 2C; *, p < 0.05; **, p < 0.01; n = 3–5).

Consistent with an increased expression of TRPM7 protein, whole-cell patch-clamp recordings demonstrated that HG treatment for 48 h significantly increased TRPM7-like currents in NS20Y cells (control, −8.6 ± 8.7 pA/pF; HG, −15.64 ± 13.6 pA/pF; *, p < 0.05; n = 20 cells; Fig. 2D).

HG induces cytotoxicity in NS20Y cells

Because ERS-related molecules, such as CHOP and GRP78, were increased by HG, we examined the potential change in the expression of caspase-12, an ERS-regulated molecule. We
found that the relative level of cleaved caspase-12, which is the active form of caspase-12, was increased to 1.74 ± 0.31 (Fig. 3, A and B), suggesting that HG may induce ERS-mediated apoptotic cell death. In addition, we found that the level of cleaved caspase-3, a downstream effector, was also increased (1.51 ± 0.14; Fig. 3, A and B).

To evaluate the injurious effect of HG on NS20Y cells, we examined the effect of HG on LDH release, an index of cell injury (33, 41). NS20Y cells were cultured in 24-well plates with medium containing 25 mM (control) or 45 mM (HG) glucose for 24 or 48 h. As shown in Fig. 3C, 24–48-h incubation of NS20Y cells with HG induced a large increase of LDH release to 2.10 ± 1.07- and 3.39 ± 1.24-fold of control level, respectively (*, p < 0.05; n = 5).

The effects of high D-glucose were not mimicked by L-glucose

Hyperglycemia or HG leads to higher osmolarity. High osmolarity itself has been suggested to produce some biological effects (42–44). For example, Zhu et al. (44) found that elevated osmolarity can affect recombinant protein production in CHO cells. To examine the possibility that changes in cell viability and protein expression by HG were mediated by the change in osmolarity, L-glucose (LG), a nonmetabolizable form of glucose, was applied instead of D-glucose.

Microscopic observation revealed no significant change in cell morphology between 25 mM D-glucose (DG) and 25 mM DG + 20 mM LG–treated groups (Fig. 4A). In contrast, HG (45 mM D-glucose) exposure clearly increased cytotoxicity, as shown by dramatic deformation of cell morphology (Fig. 4A). Consistent with morphological data, there was no difference in relative LDH release between 25 mM D-glucose (DG) and 25 mM DG + 20 mM LG–treated groups, but the relative LDH release was clearly increased in the HG-treated group (***, p < 0.01; n = 8; Fig. 4B). Furthermore, compared with the 25 mM DG + 20 mM LG–treated group, the relative levels of TRPM7, Inos, Chop, and Grp78 mRNA in the HG group were significantly increased to 1.48 ± 0.14, 4.42 ± 1.45, 1.68 ± 0.53, and 1.72 ± 0.50, respectively (*, p < 0.05; n = 3–4; Fig. 4C). These findings strongly suggest that HG-induced changes in protein expression and cell viability in the present study were not mediated by the increase of osmolarity.
Effect of TRPM7 inhibition on HG-induced cell injury and ER stress

To investigate the involvement of TRPM7 in HG-induced cell injury, we first examined the effect of 2-aminoethoxydiphenyl borate (2-APB) and gadolinium (Gd³⁺), two commonly used nonspecific inhibitors of TRPM7, on HG-induced injury of NS20Y cells. As shown in Fig. 5(A and B), both 2-APB (100 μM) and Gd³⁺ (20 μM) significantly reduced HG-induced relative LDH release from 1.35 ± 0.23 and 1.48 ± 0.11 to 0.94 ± 0.10 and 1.15 ± 0.21, respectively (n = 5–6; *, p < 0.05; **, p < 0.01). The effects of TRPM7 inhibitors 2-APB (D), Gd³⁺ (E), and waixenicin A (F) on HG induced increased expression of ERS-related molecules GRP78 and CHOP, respectively (n = 4–6; *, p < 0.05; **, p < 0.01). Error bars, S.D.

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Effect of NOS inhibitor on TRPM7 expression in HG-treated cells

Our finding that the level of iNOS is largely increased in diabetic brain tissue and in NS20Y cells treated with HG suggested that increased production of nitric oxide (NO) may play a role in increased TRPM7 expression and cell injury. NO has been shown to play an important role in many physiological and pathological processes. It has been shown that high glucose can induce overproduction of NO in a variety of nonneuronal cells, including umbilical vein endothelial cells, vascular smooth muscle cells, mesangial cells, lens epithelial cells, human retinal pigment epithelium, Schwann cells, and hepatocytes (46–50). To determine whether NOS/NO plays a role in HG-mediated
changes in NS20Y cells, we examined the effect of NOS inhibition on HG-induced cell injury and the change in the level of TRPM7 expression. NS20Y cells were cultured with HG in the absence and presence of 500 \( \mu \)M N-nitro-L-arginine methyl ester (L-NAME), a commonly used inhibitor of NOS. As shown in Fig. 6A, HG significantly increased NO production, which was inhibited by L-NAME (*, \( p < 0.05 \); \( n = 4 \)). B, cell injury was analyzed with LDH release (\( n = 5–6 \)). C, the level of Trpm7 mRNA was examined with quantitative PCR. The corresponding bar graph shows the relative expression of Trpm7 normalized to Gapdh; \( n = 3 \); *, \( p < 0.05 \) versus HG. Error bars, S.D.

Figure 6. NOS inhibitor L-NAME inhibits HG-induced NO production, cellular injury and TRPM7 expression in NS20Y cells. Cells were cultured with HG with or without L-NAME for 48 h. A, supernatants analyzed for levels of total nitrite/nitrate as a measure of NO (\( n = 4 \); *, \( p < 0.05 \)). B, cell injury was analyzed with LDH release (\( n = 5–6 \)). C, the level of Trpm7 mRNA was examined with quantitative PCR. The corresponding bar graph shows the relative expression of Trpm7 normalized to Gapdh; \( n = 3 \); *, \( p < 0.05 \) versus HG. Error bars, S.D.

Effect of TRPM7 silencing on HG-induced changes of ERS-associated molecules and cytotoxicity

To gain more insight into the role of TRPM7 in HG-induced cellular injury, a TRPM7-specific siRNA was employed to silence TRPM7 channels. Cells were transfected with either Trpm7 siRNA (against mouse Trpm7 corresponding to coding region 5152–5172, GenBank™ code AY032951) or control nontargeting siRNA and cultured in the absence or presence of HG for 48 h. We determined whether silencing TRPM7 affects HG-induced changes of ERS-associated molecules in NS20Y cells. Quantitative PCR confirmed that the relative level of Trpm7 mRNA was significantly down-regulated to 0.14 \( \pm \) 0.05 (Fig. 8A; *, \( p < 0.05 \); \( n = 3 \)). The relative levels of Chop and Grp78 were decreased to 0.44 \( \pm \) 0.08 and 0.60 \( \pm \) 0.03, respectively (**, \( p < 0.01 \)). As expected, the relative level of TRPM7 protein was decreased to 0.38 \( \pm \) 0.15 in cells treated with Trpm7 siRNA (Fig. 8B; **, \( p < 0.01 \); \( n = 11–12 \)).
Compared with control siRNA-treated cells, the levels of cleaved caspase-12, cleaved caspase-3, and CHOP proteins were significantly decreased to 0.38 ± 0.14, 0.82 ± 0.10, and 0.83 ± 0.09 in Trpm7 siRNA-treated cells (Fig. 8B and C; *, p < 0.05; **, p < 0.01; n = 3–12). Consistent with the reduction of TRPM7 expression, TRPM7-like currents were reduced in TRPM7 siRNA treated cells (Fig. 8D). The density of TRPM7-like currents was decreased from −10.48 ± 3.38 to −4.77 ± 2.42 in cells without HG treatment and from −27.70 ± 16.52 pA/pF to −11.42 ± 5.65 pA/pF in HG-treated cells (Fig. 8D; **, p < 0.01; n = 19–22 cells). As expected, transfection with Trpm7 siRNA attenuated HG-induced increase of LDH release from 1.55 ± 0.44 to 1.05 ± 0.24 (Fig. 8E; *, p < 0.05; n = 5–6).

We also examined the effect of another Trpm7 siRNA (against mouse coding region 1630–1650, GenBank™ code
NM_021450.2). It showed a similar knockdown efficiency in suppressing TRPM7 protein expression (Fig. S2). As expected, ERS-associated molecule CHOP was also significantly decreased (Fig. S2), excluding the possibility that the siRNA-induced outputs were derived from the off-target effect.

Effect of TRPM7 overexpression on cytotoxicity, apoptosis, and ERS

We further determined whether TRPM7 overexpression has an opposite effect on cytotoxicity/apoptosis and ERS compared with TRPM7 knockdown or inhibition. As shown in Fig. 9, transient transfection of NS20Y cells with pcDNA4-TO-TRPM7 for 48 h caused an increased cell injury, as shown by dramatic change in cell morphology (Fig. 9A). It also increased the level of ERS-related proteins, including CHOP and GRP78, and apoptosis-related proteins, including cleaved caspase-12 and cleaved caspase-3 (Fig. 9B and C); *, p < 0.05; **, p < 0.01; n = 3–6). Furthermore, in HEK-293 cells stably transfected with TRPM7, induced overexpression of TRPM7 for 48 h also resulted in increased cell injury and LDH release (Fig. 9, D and E; 1.87 ± 0.33; **, p < 0.01; n = 9) and increased level of cleaved caspase-12 and cleaved caspase-3 (Fig. 9F and G); *, p < 0.05; n = 6). These findings, together with the TRPM7 knockdown and inhibition data, support the hypothesis that TRPM7 plays an important role in HG-induced cytotoxicity through ERS-associated pro-apoptotic activity.

Role of TRPM7 kinase in TRPM7-mediated ERS and cytotoxicity

TRPM7 combines structural elements of both an ion channel and a protein kinase (51). Some studies have suggested that the kinase domain/activity is essential for the channel function, whereas others reported that TRPM7 kinase does not influence the channel function (51). To determine whether the kinase domain/activity is involved in TRPM7-mediated ER stress and cytotoxicity, we examined the ERS and cell injury in HEK-293 cells stably transfected with human TRPM7 cDNA for 48 h with 1 μg/ml tetracycline (Fig. 9, D and E). HEK-293 cells stably transfected with human TRPM7 cDNA were treated with 1 μg/ml tetracycline for 48 h for induced expression of TRPM7. E, LDH assay showing that TRPM7 overexpression resulted in injury of HEK-293 cells. n = 9; **, p < 0.01, unpaired t test. Representative blots (F) and summary data (G) show the expression of TRPM7, cleaved caspase-3, and cleaved caspase-12, without or with TRPM7 overexpression. n = 6. *, p < 0.05. Error bars, S.D.
As shown in Fig. 10, induced expression of both TRPM7 mutants for 48 h by tetracycline caused significant cytotoxicity (Fig. 10, A and E). The LDH release was increased to 1.29 ± 0.07- and 1.47 ± 0.06-fold in cells with overexpression of TRPM7-Δkinase and TRPM7-K1648R, respectively (Fig. 10B and F), **, p < 0.01; n = 4 and n = 9). The expression of GRP78 and CHOP was also increased by overexpression of TRPM7-Δkinase or TRPM7-K1648R (Fig. 10, panels C and D and panels G and H). These data suggest that TRPM7’s terminal kinase may not play a critical role in TRPM7-mediated ERS and cytotoxicity. Future studies will consider overexpressing TRPM7 with channel pore mutations to provide additional evidence that the ion-conducting function of TRPM7 is indeed involved in HG-mediated ERS and neuronal cell injury.

**Effect of NOS inhibitor on TRPM7 expression and ERS in brain tissues isolated from the insulin-deficiency model of diabetic mice**

Our in vitro and in vivo data have demonstrated that diabetes/hyperglycemia increase the expression of TRPM7, iNOS, and ERS. Our in vitro data also suggest a signaling cascade: iNOS/TRPM7-ERS. To provide some evidence in vivo, we investigated the effect of l-NAME, the nitric-oxide synthase inhibitor, on TRPM7 expression and ERS-related molecules in an insulin-deficiency model of diabetes. Our results showed that l-NAME injection (400 mg/kg, intraperitoneally) for 3 days significantly decreased the level of TRPM7 and ERS markers GRP78 and CHOP in cortical tissue, compared with those in cortical tissue of diabetic mice with saline treatment (Fig. 11; *, p < 0.05; n = 4–5). These results further suggest that NOS/NO play a critical role in HG/hyperglycemia-mediated up-regulation of TRPM7 and ERS.

**Discussion**

In the present study, the effects of diabetes and HG on expression of TRPM7, NOS, CHOP, and GRP78 were investigated by using an in vitro cell culture model and in vivo diabetic mice. We show, for the first time, that TRPM7 channels are implicated in HG-induced ERS and resultant cytotoxicity in a neuronal cell line. We demonstrated that 1) HG induces injury and apoptosis of NS20Y cells; 2) TRPM7, iNOS, CHOP, and GRP78 expressions are increased both in HG-treated neuronal cell line in vitro and in cortical tissues of diabetic mice in vivo; and 3) pharmacological inhibition and gene knockdown of TRPM7 channels ameliorate HG-induced ERS and cellular damage. In contrast, overexpression of TRPM7 enhances the expression of ERS-related molecules. Overexpression of TRPM7 increases ERS and cellular injury independent of the TRPM7’s terminal kinase. Our studies also suggest a signaling cascade underlying HG-induced cell injury via iNOS-TRPM7-ERS.

Optimal survival and neurite growth of cortical neurons require 25 mM basal glucose, reflecting the fact that neurons have high metabolic rates (3, 6). Treatment of NS20Y cells with HG (e.g. 45 mM DG, 48 h), however, increased LDH release, demonstrating HG-mediated cytotoxicity. Oxidative stress is associated with diabetic/hyperglycemic conditions and cell injury. A high amount of NO, for example, produced particularly by iNOS, is involved in pro-inflammatory reactions and tissue damage (52). In the current studies, we examined potential change in NOS expression under HG conditions. We showed that the level of iNOS is largely increased by HG treatment. Consistent with the involvement of NOS in HG-medi-
TRPM7 in hyperglycemia-mediated ER stress

**Figure 11.** NOS inhibitor L-NAME inhibits hyperglycemia-induced expression of TRPM7 and ERS-related molecules in cortical tissue from an insulin-deficiency model of diabetic mice. Diabetic mice were treated with L-NAME (400 mg/kg intraperitoneally daily) or saline for 3 days at day 5 following intravenous injection of alloxan. Representative blots (A and summary data (B–D) show the decreased expression of TRPM7, GRP78, and CHOP by L-NAME (n = 4–5), *p < 0.05 versus saline. Error bars, S.D.

- **A** shows the molecular weight of TRPM7, GRP78, CHOP, and β-actin.
- **B** demonstrates the decreased expression of TRPM7 by L-NAME.
- **C** and **D** illustrate the decreased expression of GRP78 and CHOP by L-NAME.

**ERS and resultant apoptosis in a neuronal cell line.** The detailed mechanism underlying TRPM7-mediated ERS is still unclear. Whether it induces structural changes within the ER or through calcium/magnesium/zinc signaling or other pathways involved in apoptosis needs to be further examined. It would also be interesting to examine the relationship between TRPM7 and the earlier markers of UPR, such as splicing of XBP1, PERK phosphorylation, and cleavage of ATF6. Nevertheless, our findings that extracellular Zn^{2+}-chelating agent Ca^{2+}.EDTA, but not Ca^{2+}/Mg^{2+}-chelating agent EGTA, decreased HG-induced GRP78 expression, suggesting that zinc influx through TRPM7 channels, at least partly, contributes to HG-induced ERS (see Fig. S3 (A–C)).

In the present study, we focused on changes in the expression level of iNOS, TRPM7, ERS stress marker, and caspase cleavage at 48 h after HG treatment. Future experiments will examine the detailed time course for the changes of these molecules (e.g., at 12 and 24 h after HG treatment) and determine whether the time course of expression of these molecules correlates well with cell injury and whether cell injury is blocked by caspase inhibitors, such as benzylxycarbonyl-VAD-fluoromethyl ketone.

It has been reported that excessive NO generation and resultant reactive oxygen species (ROS) are involved in various pathophysiological processes (52). It has also been demonstrated that ROS stimulates TRPM7 activity and that oxidative stress-induced cytotoxicity can be blocked by inhibition of TRPM7 (22, 53). In the present study, we found that Inos mRNA and protein were up-regulated in HG-treated NS20Y cells. Because L-NAME, an inhibitor of NOS, reduced HG-induced cell injury, activation of iNOS is probably involved in HG-induced injury of NS20Y cells. Moreover, we showed that L-NAME administration significantly decreased TRPM7 expression, indicating the possibility that NOS/NO contributes to HG-induced injury, at least in part, by increasing the expression/activity of TRPM7 channels. The finding that TRPM7 knockdown ameliorates NO donor-induced cell injury (see Fig. S4 (A and B)) further supports the involvement of TRPM7 in NO/ROS-mediated cell injury. The exact relationship between NO/ROS production and TRPM7 expression or activation is probably complicated and maybe cell type–dependent. In human umbilical vein endothelial cells, we demonstrated in our previous study (31) that HG treatment suppresses eNOS expression and NO production but increases ROS production in a TRPM7-dependent manner. Therefore, additional studies delineating the detailed relationship between NO/ROS production and TRPM7 expression/activation in different cell types will be important for advancing knowledge in the field.

NO may cause a biological/pathological effect directly or through peroxynitrite by interacting with superoxide anion. Our findings that Tiron, a superoxide anion scavenger, and uric acid, a peroxynitrite scavenger, potently inhibited HG-induced TRPM7 expression suggest that peroxynitrite is involved in HG-induced TRPM7 expression (Fig. S5).

The detailed mechanisms of how NO production increases TRPM7 expression warrant further investigation. In the current study, we showed a clear increase in NO production and TRPM7 expression 48 h after HG treatment. Future studies...
may compare the detailed time course of NO production with Trpm7 mRNA expression and determine whether an increase of NO production occurs before TRPM7 induction. In addition, it will be interesting to examine changes in Trpm7 mRNA level by HG treatment in the presence of cycloheximide to determine whether the transcription of Trpm7 mRNA is increased or the degradation of Trpm7 mRNA is decreased by HG treatment. In summary, our current findings suggest that TRPM7, iNOS, and ERS participate in HG-induced injury of NS20Y cells. As for the proposed sequence of events, it is likely that HG initially enhances iNOS expression, and ROS generated by iNOS subsequently increases the expression and/or activity of TRPM7 channels. Activation of TRPM7 channels promotes ERS responses and resultant caspase activation, facilitating apoptotic cell injury. Although the mechanism of how TRPM7 regulates ERS is not fully understood, our findings that TRPM7 channels stimulate ERS, combined with our previous report that inhibition of TRPM7 protects neurons from various injurious stimuli, raise the possibility that targeting TRPM7 channels might prove to be a novel therapeutic strategy for brain injury under hyperglycemic/diabetic conditions.

Experimental procedures
Reagents and antibodies

2-APB (catalog no. D9754), gadolinium (Gd³⁺; catalog no. G7532), I-NAME (catalog no. N5751), uric acid (catalog no. U2625), EDTA calcium disodium salt (catalog no. ED25C), EGTA (catalog no. E4378), and diethylaminoethyl/nitric oxide adduct (DETA/NO, catalog no. D185) were purchased from Sigma. Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), transfection reagent Lipofectamine™ RNAiMAX, and primers for quantitative PCR were purchased from Invitrogen. Rabbit polyclonal antibodies against TRPM7 (catalog no. ab85016), mouse anti-TRPM7 antibody (catalog no. ab135817), mouse anti-TRPM7 antibody (catalog no. ab85016), and β-actin (catalog no. ab8227) were purchased from Abcam. Rabbit polyclonal antibodies against GRP78/BiP (catalog no. 3183), mouse monoclonal antibody CHOP (catalog no. 2895), rabbit anti-caspase-12 (catalog no. 2202), and anti-cleaved caspase-3 (catalog no. 9664) antibodies were purchased from Cell Signaling. Mouse monoclonal antibody against HA tag (catalog no. M180-3) was purchased from MBL Life Science. The nitric oxide assay kit (Fluorometric) was purchased from Cell Oxidant. Rabbit polyclonal antibodies against GRP78/BiP (catalog no. 3183), mouse monoclonal antibody CHOP (catalog no. 2895), rabbit anti-caspase-12 (catalog no. 2202), and anti-cleaved caspase-3 (catalog no. 9664) antibodies were purchased from Cell Signaling. Mouse monoclonal antibody against HA tag (catalog no. M180-3) was purchased from MBL Life Science. The nitric oxide assay kit (Fluorometric) was purchased from Cell Oxidant.

Cell culture and transfection

NS20Y cells, a neuronal cell line derived from mouse neuroblastoma (40, 55), were cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose supplemented with 10% FBS and antibiotics. Culture medium was changed every 2 days. Knockdown of TRPM7 was performed as described previously (31). Briefly, siRNA against mouse TRPM7 corresponding to coding region 5152–5172 (Trpm7 siRNA, GenBank™ code AY032951) was synthesized by Invitrogen. Cells were transfected with siRNA using transfection reagent Lipofectamine™ RNAiMAX according to the manufacturer’s instructions. Nontargeting siRNA was used as a control (catalog no. AM4611). Cells were used for experiments 2–3 days after transfection. pcDNA4/TO encoding TRPM7 channels was provided by Carsten Schmitz’s laboratory (University of Colorado). NS-20Y Cells were transfected with 2.5 μg of plasmid using transfection reagent Lipofectamine® 3000 (Invitrogen) according to the manufacturer’s instructions.

Human embryonic kidney (HEK-293) cells, stably transfected with the HA-tagged human TRPM7 construct, or phosphotransferase activity-deficient point mutants of TRPM7, K1648R, as well as the Δ-kinase mutant were cultured in minimum essential medium supplemented with 10% FBS and antibiotics. For the induction of expression of TRPM7 or TRPM7 mutants, the cells were treated with 1 μg/ml tetracycline for 48 h, as described in our previous studies (56).

Cytotoxicity assay

Quantitative assessment of cell injury was performed by measurement of lactate dehydrogenase (LDH) released into the culture medium as described previously (31, 33). Cells grown on 24-well plates were washed with PBS and treated with 550 μl of the above-mentioned culture medium containing either 25 mM (control), 45 mM d-glucose (HG), or 25 mM d-glucose plus 20 mM l-glucose (DG + LG), respectively. 50 μl of medium was immediately taken from each well and placed into a 96-well plate for background LDH measurement. At different time points following the treatment, 50 μl of medium was taken from each well for LDH measurement. At the end of each experiment, cells were lysed by adding 50 μl of medium containing Triton X-100 (final concentration, 0.5%) and incubated for 30 min at 37 °C. 50 μl of supernatants were withdrawn from each well for maximal LDH measurement. 50 μl of assay reagent from the cytotoxicity detection kit (Roche Diagnostics) was added to each 50-μl sample and mixed. 20 min later, the absorbance at 492 nm was measured by spectrometer (Spectra Max Plus, Molecular Devices) and subtracted by the absor-
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Table 1

| Forward | Reverse | Size |
|---------|---------|------|
| Gapdh   | CTTTGGAAGGCAATGAGCCGATGA | 105  |
| Trpm7   | CTATCCAGTCTCCACAGTCCA   | 399  |
| Inos    | GTCTTCCTTCTGCGTTCTCT    | 101  |
| Chop    | TTTCCCTGGCTGTTGCGTCT    | 340  |
| Gpr78   | GCACTGGGAGGCTGCTGCT     | 398  |

Nitric oxide assay

At the end of the treatment, nitric oxide generated in the culture medium was quantitated by measurement of the total nitrite/nitrate using the nitric oxide assay kit (ab65327, Abcam) following the manufacturer’s protocol. Briefly, 75 µl of supernatant of the culture medium was transferred to a 96-well plate, followed by the addition of 5 µl of enzyme cofactor and 5 µl of nitrate reductase. The plate was incubated at room temperature for 3 h. 5 µl of enhancer was then added to each well and incubated for 30 min. 5 µl of the DAN probe was added to each well, the plate was incubated for 10 min, and 5 µl of NaOH was added before final incubation for 10 min. Fluorescence was determined with a microplate reader (SpectraMax M5) at excitation/emission = 360/450 nm. Concentrations of NO were then derived from the standard curve.

Quantitative real-time PCR

Real-time PCR was performed as described previously (32). Total RNAs were extracted with an RNA purification kit (Qiagen) and reverse-transcribed to cDNA using the Superscript® first-strand synthesis system (Invitrogen). Quantitative PCR was performed using SsoAdvanced™ SYBR® Green supermix (Bio-Rad) with a C1000™ thermal cycler (Bio-Rad). PCR was initiated at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 61 °C for 45 s. To validate the quality of PCR products, melting curve analysis was carried out from 65 to 95 °C with a heating rate of 0.1 °C/s. All reactions were carried out in duplicates or triplicates, and the average Ct values were calculated. Ct methods were used to analyze the results (32). The Gapdh gene was used as an internal control. GAPDH is a key enzyme involved in glycolysis. The Gapdh gene is expressed at high levels in almost all tissues. As a housekeeping gene, the levels of Gapdh expression in cells or tissues of the same species are generally constant. In the present study, the absolute Gapdh levels in different groups showed no difference. PCR primer sequences are listed in Table 1.

Western blotting

Western blotting was performed as described previously (32). Cells cultured in 35-mm dishes were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease, and phosphatase inhibitor mixture). RIPA lysis and extraction buffer (Thermo Fisher Scientific) was used to extract the protein from cortex tissue. Cortex tissues were transferred to a homogenizer with RIPA buffer containing protease inhibitors. 500 µl of RIPA buffer was added for approximately every 10 mg of tissue. Tissues were homogenized thoroughly and kept on ice for 30 min. After centrifugation at 13,000 X g for 30 min at 4 °C, the lysates were collected. Bradford reagent (Bio-Rad) was used to assay the protein concentration. The aliquots were then mixed with Laemmli sample buffer and boiled at 95 °C for 5 min. 30 µg of total protein was loaded for each lane. The samples were resolved by 10% SDS-PAGE, followed by electrotransfer to polyvinylidene difluoride membranes. For visualization, blots were probed with antibodies against TRPM7 (1:1000), cleaved caspase 3 (1:1000), cleaved caspase 12 (1:1000), CHOP (1:1000), GPR78 (1:500), or β-actin (1:2000), followed by detection using horseradish peroxidase–conjugated secondary antibodies (1:2000; Cell Signaling), and developed using an ECL kit (Millipore). The intensity of the protein band was quantified with ImageJ software (National Institutes of Health).

Electrophysiology

Whole-cell voltage-clamp recordings were performed as described (57). Patch electrodes were fabricated from borosilicate capillary tubing of 1.5 mm in diameter (WPI) using a vertical puller (PP-83, Narishige) and had resistances of 3–4 megaohms when filled with electrode solution (see below). Currents were recorded using an Axopatch 200B amplifier with pCLAMP version 10 software (Axon Instruments). They were filtered at 2 kHz and digitized at 5 kHz using Digidata 1322A. Data were eliminated from statistical analysis when access resistance was >10 megohms or leak current was >100 pA at −60 mV. A multibarrel perfusion system (SF-77B, Warner Instruments) was used to achieve a rapid exchange of external solutions. Standard ECF contained 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 10 mM glucose (pH 7.4 adjusted with NaOH; 320–335 mosm). Patch electrodes contained 140 mM CsF, 10 mM HEPES, 1 mM CaCl₂, 11 mM EGTA, 2 mM tetraethylammonium chloride (pH 7.3 adjusted with CsOH, 290–300 mosm). All experiments were done at room temperature (22–24 °C).

Statistical analysis

Data are presented as the mean ± S.D. Student’s t test was used for statistical comparisons of data from experiments with only two conditions. For experiments with more than two conditions, data were subjected to one-way analysis of variance followed by Bonferroni’s test. A p value of <0.05 was considered significant.
Author contributions—Y. H. conducted most of the experiments, analyzed the results, and wrote most of the paper. T.-D. L. conducted whole-cell voltage-clamp and TRPM7 overexpression experiments and helped revise the manuscript. K. I. helped to conduct TRPM7 silencing experiments. T. Y. performed most of the animal experiments. F. D. H. and A. F. isolated and processed the specific TRPM7 inhibitor waixenicin A and helped revise the manuscript. J. L. and Z.-G. X. conceived the idea for the project and wrote the paper with Y. H.

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