Contribution of TNF receptor 1 to retinal neural cell death induced by elevated glucose

G.N. Costa, J. Vindeirinho, C. Cavadas, A.F. Ambrósio, P.F. Santos

Abstract

Diabetic retinopathy (DR), a leading cause of vision loss and blindness among working-age adults, holds several hallmarks of an inflammatory disease. The increase in cell death in neural retina is an early event in the diabetic retina, preceding the loss of microvascular cells. Since tumor necrosis factor-α (TNF-α) has been shown to trigger the death of pericytes and endothelial cells as well as the breakdown of the blood–retinal barrier, we set out to investigate whether TNF-α acting through tumor necrosis factor receptor 1 (TNFR1), the major receptor responsible for mediating TNF-induced cell death, could also be responsible for the early neuronal cell death observed in DR. We used retinal neural cell cultures exposed to high glucose conditions, to mimic hyperglycaemia, and evaluated the contribution of TNFR1 in neuronal cell death. TNFR1 was found to be present to a great extent in retinal neurons and the levels of this receptor were found to be altered in cells cultured in high glucose conditions. High glucose induced an early decrease in cell viability, an increase in apoptosis and a higher immunoreactivity for the cleaved caspase-3, indicating a high glucose-induced caspase-dependent cell death. These observations were correlated with an increase in TNF-α expression. Nonetheless, inhibiting the activation of TNFR1 was sufficient to prevent the decrease in cell viability and the increase in retinal cell death by apoptosis. In conclusion, our data indicate that TNF-α acting through TNFR1 is responsible for the high glucose-induced cell death and that blocking the activity of this receptor is an adequate strategy to avoid cell loss in such conditions.

Introduction

Diabetic retinopathy (DR), one of the most common complications of diabetes, is the leading cause of legal blindness among working-age adults. Traditionally this disease is characterized by microvascular abnormalities, such as blood–retinal-barrier breakdown and vascular occlusion, which ultimately lead to retinal tissue ischemia and proliferation of new blood vessels. However, considerable evidence has been gathered showing that the neural retina is also affected in diabetes. Not only alterations in the electroretinograms of diabetic patients have been observed, but also apoptosis in the neural retina and impairment in glutamatergic signalling in diabetic retinas (reviewed in Barber et al., 2011). Many of these alterations are found in the early stages of diabetes (Barber et al., 1998; Hammes et al., 1995; Rungger-Brandle et al., 2000), preceding the microvascular alterations characteristics of diabetic retinopathy.

Besides findings evidencing the neurodegenerative events of DR, current reports have also established inflammation as crucial in the progression of the disease. As reported in Gaucher et al. (2007), microglia undergoes activation prior to ganglion cell death. Furthermore, the anti-inflammatory drug minocycline has been found to prevent caspase-3 activation in the retina during experimental diabetes (Krady et al., 2005). Compelling evidence for a role played by cytokines such as the vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in addition to the process of leukostasis (Abu el Assar et al., 1992; Sone et al., 1997), shows that DR presents several hallmarks of a chronic inflammatory disease (Adamis and Berman, 2008; Kern, 2007).

TNF-α is a pleiotropic cytokine and a key molecule in inflammatory signalling. In the central nervous system (CNS), microglia, astrocytes and infiltrating leukocytes are the primary source of TNF-α (Feuerstein et al., 1994). Moreover, in the CNS, this cytokine has been shown to mediate leukocyte adhesion (Probert et al., 1995), vascular leakage and endothelial cell death (Koizumi et al., 2003). TNF-α interacts in a membrane bound form with two distinct receptors, tumor necrosis factor receptor 1 (TNFR1; CD120a; p55) and tumor necrosis

Abbreviations: DR, diabetic retinopathy; HG, high glucose; TNFR1, tumor necrosis factor receptor 1; sTNFR1, soluble tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2; TACE, tumor necrosis factor-α converting enzyme.

* Corresponding author at: Center for Neuroscience and Cell Biology, Dept. of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal. Fax: +351 239 822 776, E-mail address: pfsantos@ci.uc.pt (P.F. Santos).

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doi:10.1016/j.mcn.2012.04.003
factor 2 (TNFR2: CD120b: p75), while its proteolitically processed soluble form (a homotrimeric assembly of 17 kDa soluble proformers) predominantly signals through TNFR1. Hence, TNF-α can perform a pro-inflammatory/pro-apoptotic role, when signaling through TNFR1, as well as an anti-inflammatory/pro-survival function, when acting on TNFR2. Upon ligand binding, the TNFRs intracellular domains recruit multiple adaptor proteins such as TRADD, FADD, RIP1, TRAF1 and TRAF2 which in turn mediate the activation of several effector proteins, including NF-kB and caspase-8 (reviewed in Wajant et al., 2003). Much attention has been cast on TNFR1 due to its key role in inflammation and distinctive capacity to elicit cell death through apoptotic cascades initiated by its Death Domain (DD) (Boldin et al., 1996).

Among numerous effects promoted by TNF-α in the CNS, particularly in the retina, several are intimately related to alterations observed in DR, such as: increased endothelial cell permeability (Avelleira et al., 2010), breakdown of the blood–retinal barrier (BRB) (Luna et al., 1997), induction of leukocyte adhesion (Koizumi et al., 2003) and activation of microglial cells (Park et al., 2007). In addition, independent of a broader inflammatory response and/or tissue edema, TNF-α released by microglia can have direct neurotoxic effects (Taylor et al., 2005). Studies in experimental diabetes, as well as in human diabetic patients, have shown that TNF-α plays a role in the pathogenesis of DR (El-Remessy et al., 2006; Joussen et al., 2002; Yuuki et al., 2001), but the contribution of this cytokine to the neuronal cell death observed in the retina during diabetes has remained unexplored.

In the present work, we used retinal cell cultures maintained in a high glucose concentration medium as a model to study retinal cells in a situation of hyperglycaemia, simulating the conditions found during diabetes. We found that exposure to high glucose resulted in an early decrease in cell viability as well as an increase in TNF-α expression. This event was also followed by an increase in apoptosis. Thus, we further investigated whether the high glucose-induced increase in retinal cell death could result from TNF-α signaling through TNFR1, the most likely candidate for mediating the noxious effects of TNF-α. Our results show that blockade of TNFR1, which is conspicuously expressed in retinal neurons, is capable of preventing the increase in cell death induced by high glucose. These results support a decisive role for TNF-α and TNFR1 in the pathogenesis and outcome of DR.

**Results**

**TNFR1 is enriched at neuronal synaptic terminals**

Given the importance of TNFR1 as the major receptor responsible for the TNF-α induced cell death, the localization of this receptor in the heterogeneous population of retinal cells present in our culture model was determined by immunocytochemistry using confocal microscopy. Fig. 1 shows an immunofluorescence image of TNFR1 (Fig. 1A) and the neuronal synaptic marker Synaptophysin (Syn) (Fig. 1B). The merged image (Fig. 1C) reveals that TNFR1, despite being present in cell perikarya (arrows) and having a ubiquitous distribution through the different cell types present in these mixed cultures (i.e. amacrine cells, astrocytes, Müller cells, microglia and photoreceptors; data not shown), is located to a great extent in neuronal synapses. This can be seen by the strong colocalization with Syn, clearly defined by the punctated yellow staining (arrowheads, Fig. 1C). A positive correlation was found between the immunoreactivity for Syn and TNFR1, as evaluated by the Pearson's correlation coefficient ($r = 0.53 \pm 0.01$; $n = 12$). Moreover, the overlap of about 60% of the Syn staining with TNFR1, measured by the Manders coefficient ($M_1 = 0.62 \pm 0.03$; $n = 12$) confirms that synapses are highly enriched in TNFR1. The distribution pattern of this receptor shows that retinal neurons should be particularly sensitive to TNF-α given the high-affinity of TNFR1 for this cytokine (Grell et al., 1998) and the important role played by this receptor in TNF signaling.

**TNFR1 levels are altered in retinal cells exposed to high glucose**

Hyperglycaemia is considered the main factor in diabetes behind the development of DR. Therefore, in the current study, cultured retinal neural cells were exposed to high glucose (30 mM; HG) to mimic hyperglycaemic conditions, in order to investigate whether prolonged exposure to HG leads to changes in the TNF signaling system. Mannitol (25 mM) was used as an osmotic control. Since both receptors, TNFR1 and TNFR2, play a central role in the outcome of TNF-α signaling, the protein levels of each receptor was investigated by Western blot. After 7 days of culturing cells in HG, the protein levels of TNFR1 decrease around 40%, to 60.7±9.4% of control (p<0.01; Fig. 1D). This reduction is not the result of an osmotic effect, as TNFR1 protein levels were not different in cells exposed to mannitol (100.1±14.1% of control). This effect seems to be specific for the TNFR1 since no differences were found when TNFR2 levels were assessed in the same conditions (97.4±11.7% and 99.8±11.3% of control for cells exposed to high glucose and mannitol, respectively; Fig. 1E).

In order to determine whether a decrease in TNFR1 expression could account for the reduced protein levels of TNFR1, the TNFR1 mRNA levels, as well as TNFR2 mRNA levels, were evaluated by quantitative RT-PCR (Fig. 1F and G). Concerning the expression of TNFR1, after 7 days of incubation, no significant differences were found in the mRNA transcript levels among the conditions tested (1.21±0.13 and 1.22±0.20 mRNA levels relative to control, for HG and mannitol respectively). As for the expression of TNFR2, a highly regulated receptor, also no differences were found between control and HG.

**The downregulation of TNFR1 is not mediated by TACE/ADAM17-dependent receptor shedding**

It is known that the action of the TACE/ADAM17 metalloprotease, a member of the ADAM (proteins containing a disintegrin and metalloprotease domain) protein family can lead to the release of TNF-receptors from the cell membrane to the extracellular medium (Reddy et al., 2000). Since we have observed a decrease in the TNFR1 levels in HG-exposed retinal cell cultures, with no definite evidence for a decrease in TNFR1 mRNA expression, we investigated whether TACE protein levels and activity show any alteration due to HG that could account for the differences found in TNFR1 levels. In this regard, the levels of the soluble tumor necrosis factor receptor 1 (sTNFR1) were also measured in the cell culture medium.

The protein levels of TACE were not different among the three conditions tested (97.4±4.8 and 99.84±5.0% of control, for HG and mannitol, respectively, Fig. 2A). Several enzymes belonging to the ADAMs protein family, such as ADAM10, ADAM12 and particularly TACE/ADAM17, have their enzymatic activity regulated by a prodomain of which removal is necessary for ADAMs to acquire proteolytic activity (Milla et al., 1999). Nevertheless, evidence has been gathered showing an inducible activity of TACE, possibly requiring activation by protein kinase C (Reddy et al., 2009). Since TACE can be highly regulated, as shown by the readily enhancement of sheddase activity upon PMA stimulation (Weskamp et al., 2004), the activity of this enzyme was evaluated using a FRET peptide substrate associated with 5-FAM. Fig. 2B shows the activity of TACE plotted as the amount of cleaved fluorescent probe (5-FAM) vs time, of control, HG and mannitol cultured cells. No differences were found either in terms of total amount of fluorescent product produced (39.4±7.2, 40.0±6.1 and 40.8±10.3 pmol of cleaved 5-FAM for control, HG and mannitol respectively) or in terms of the increase rate of cleaved substrate.

In addition, in high glucose cultured cells the levels of sTNFR1 in the cell culture medium were found to be significantly smaller than those observed in control conditions, contrary to what would be expected if the process of receptor shedding was mediating the decrease in the TNFR1 levels. The concentrations of sTNFR1 measured in the cultured medium were 12.5±1.0, 8.3±0.2 and 12.3±1.1 pg/ml for control,
HG and mannitol conditions, respectively (p < 0.05; Fig. 2C). Taken together, these observations exclude the possibility that the high glucose-induced reduction in TNFR1 levels results from an increase in receptor shedding by the metalloprotease TACE.

High glucose concentration leads to a decrease in retinal cell viability and induces apoptosis

Previous studies from our laboratory have already shown that high glucose (HG) decreases retinal cell viability, after 7 days of incubation (Santiago et al., 2007). In the present study, we found that culturing cells in HG exerts a similar effect even for shorter periods of incubation. As early as 3 days exposure to HG, there was a small but significant decrease in cell viability (96.3 ± 0.6% of control, p < 0.05; Fig. 3A), while at 2 days no differences were found (100.7 ± 1.2% of control), thus, no prior time points were investigated. A relatively constant decline in cell viability was found for longer incubation periods in HG, reaching a minimum of 90.9 ± 1.0% of control at the last time point studied, 7 days (p < 0.001). The cells cultured in the presence of 25 mM mannitol, used as an osmotic control, showed no sign of reduced viability throughout the several time point studied. Since a more pronounced effect of HG in MTT reduction was found for longer exposure periods, further cell viability studies were performed after 7 days of incubation.

The outcome of HG exposure was also observed in terms of apoptotic cell death. As reported previously (Santiago et al., 2007), we found that after 7 days of incubation with HG the number of apoptotic nuclei detected by TUNEL assay was significantly increased, around 50% higher than control (19.3 ± 0.7 cells/field in HG compared to 12.7 ± 1.0 and 13.5 ± 0.9 in control and mannitol, respectively; p < 0.001), as displayed in Fig. 3B. This result concurs with the decreased cell viability found with the MTT assay.

Caspase-3 activity is increased due to elevated glucose

Cells can undergo apoptosis in a caspase-dependent manner, having caspase-8 and ultimately caspase-3 as the main effectors of apoptosis, but also through action of the apoptosis inducing factor (AIF) and endonuclease G, in a mechanism independent of caspase. To address the nature of the apoptotic signals resulting from elevated glucose, we investigated whether the levels of activated caspase-3, the executioner caspase, were affected by HG. For this, an antibody for the cleaved caspase-3 (Asp175), i.e. the activated form of this protease, to quantify its immunoreactivity in our retinal cultures. Notably, under HG
conditions retinal cell cultures showed a higher number of cleaved caspase-3 positive cells (Fig. 4B and C), in comparison to control. As our analysis shows (Fig. 4A), HG induced an increase of 50% in the number of cells presenting the activated form (9.6 ± 0.5 and 14.5 ± 0.6 cleaved caspase-3 positive cells/field, for control and HG, respectively; p < 0.01), an increase comparable to the number of apoptotic nuclei found by TUNEL. Since TNF-α mainly promotes apoptosis through caspase activation, these results support a role for this cytokine in the HG-induced retinal cell death.

High glucose induces photoreceptor and bipolar cells apoptosis

An increase in cell apoptosis could result from the death of one or several cell types present in our mixed retinal cultures (e.g. microglia, Müller cells, astrocytes, neurons and photoreceptors). In order to address this issue and identify the cells undergoing apoptosis, immunoreactivity for several cell markers was evaluated along with the fluorescein-TUNEL assay. Very few TUNEL positive cells showed immunoreactivity to neuronal markers (i.e. NeuN; [β3-Tubulin]), microglial (CD11b) or macroglial cell markers (GFAP; Vimentin), whether in control or HG (Table 1). Despite their neuronal origin, photoreceptors and most inner nuclear layer cells in the retina (e.g. bipolar cells, horizontal cells), do not show immunoreactivity for NeuN (Mullen et al., 1992). Amacrine cells, identified by the calretinin immunostaining, corresponded also to a minority of the cells undergoing apoptosis. On the other hand, rhodopsin positive cells, i.e. rod photoreceptors, were labeled by TUNEL in a greater number, particularly in HG-exposed cells (Fig. 4E and F). A significantly higher number of cells positive for both rhodopsin and TUNEL was found in HG (6.8 ± 0.7 TUNEL⁺-Rd⁺-cells/field), compared to control (2.7 ± 0.1 TUNEL⁺-Rd⁺-cells/field, p < 0.001; Fig. 4D). Moreover, a high percentage of apoptotic cells could be ascribed to rod bipolar cells, labeled with PKCo (Table 1). As shown in Fig. 4G, the number of cells double labeled for TUNEL and PKCo was, as for rhodopsin positive cells, higher in HG (3.4 ± 0.6 and 7.4 ± 1.5 TUNEL⁺-PKCo⁺-cells/field in control and HG, respectively; p < 0.05) suggesting that rod photoreceptors and rod bipolar cells are the cells most affected by the high concentration of glucose.

TNF-α expression is transiently increased in retinal cells exposed to high glucose

Early in experimental diabetes, several inflammatory mediators are found to be increased in the retina, including TNF-α which has been shown to be an important player in triggering leukocyte adhesion and
was used to identify the cells undergoing apoptosis and quantify the extent of cell death. In HG the number of TUNEL labeled with TUNEL (green) and Hoechst (white). TUNEL-labeled nuclei belonging to PKC +/−-cells do not (arrows). (E) Rod bipolar cells were immunostained with PKCo. In HG the number of PKCo +/−-cells labeled with TUNEL was higher than in control. (H and I) Control and HG cultured retinal cells, respectively, immunostained for PKC +/−-cells compared to control. (B and C) Immunoreactivity for the active caspase-3 (red fluorescence) and transmission image of control and HG retinal cell cultures, respectively. Scale bar, 50 μm. (D) Immunostaining for rhodopsin (Rd) was used to identify the cells undergoing apoptosis and quantify the extent of cell death. In HG the number of TUNEL +/−-cells was more than twice that of control. (E and F) Retinal cell cultures immunostained for rhodopsin (Rd; red) and co-labeled with TUNEL (green) and Hoechst (white), control and HG respectively. Several TUNEL +/−-nuclei pertain to cells identifiable as rods by the photoreceptor marker Rd (arrowheads), while other TUNEL +/−-cells do not (arrows). (E) Rod bipolar cells were immunostained with PKCo. In HG the number of PKCo +/−-cells labeled with TUNEL was higher than in control. (H and I) Control and HG cultured retinal cells, respectively, immunostained for PKCo (red) and co-labeled with TUNEL (green) and Hoechst (white). TUNEL-labeled nuclei belonging to PKCo +/−-cells were more numerous in HG than in control. Apoptotic cells not immunostained for PKCo are also visible (arrows). Scale bar, 20 μm. Bars represent the mean±SEM of at least three independent experiments. *p<0.05; ***p<0.001.

Fig. 4. Evaluation of the cleaved caspase-3 presence and identification of TUNEL +/−-cells. (A) The presence of active caspase-3 was detected by immunoreactivity using an antibody specific for the cleaved form of this enzyme (Asp175). Cultures exposed to HG for 7 days show 50% more cleaved caspase-3 +/−-cells compared to control. (B and C) Immunoreactivity for the active caspase-3 +/−-cells. (D) Cleaved caspase-3 +/−-cells expressed as percentage of total TUNEL +/−-cells. Data represents the amount of cells double-positive for both the cell marker and TUNEL expressed as percentage of the total TUNEL +/−-cells. Data are means±SEM of at least three independent experiments; *p<0.05, significantly different from control as determined by the Student’s t-test.

Table 1

<table>
<thead>
<tr>
<th>Cell marker</th>
<th>Control</th>
<th>High glucose</th>
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<tbody>
<tr>
<td>Rhodopsin</td>
<td>28.8 ± 2.6%</td>
<td>39.1 ± 3.2% (*1)</td>
</tr>
<tr>
<td>PKCo</td>
<td>27.8 ± 1.6%</td>
<td>42.2 ± 1.9% (*1)</td>
</tr>
<tr>
<td>NeuN</td>
<td>2.1 ± 0.8%</td>
<td>2.4 ± 0.7%</td>
</tr>
<tr>
<td>Calretinin</td>
<td>1.8 ± 0.2%</td>
<td>1.8 ± 0.4%</td>
</tr>
<tr>
<td>J3-Tubulin</td>
<td>1.7 ± 0.2%</td>
<td>0.7 ± 0.3%</td>
</tr>
<tr>
<td>CD11b</td>
<td>0.5 ± 0.1%</td>
<td>0.4 ± 0.3%</td>
</tr>
<tr>
<td>Vimentin</td>
<td>62.2 ± 3.3%</td>
<td>54.4 ± 2.3%</td>
</tr>
<tr>
<td>GFAP</td>
<td>0.8 ± 0.5%</td>
<td>3.1 ± 1.3%</td>
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Retinal cells, cultured in control and high glucose conditions, were stained for cell-specific antigens and labeled with TUNEL. Data represents the amount of cells double-positive for both the cell marker and TUNEL as expressed as percentage of the total TUNEL +/−-cells. Data are means±SEM of at least three independent experiments; *p<0.05 compared to control. **p<0.001.***p<0.001 compared to the corresponding control at each time point (two-way ANOVA followed by Bonferroni’s post test).
Selective TNFRI blockade prevents apoptosis induced by high glucose

A selective antibody against the extracellular domain of TNFR1 was used to inactivate this receptor and prevent further activity through its intracellular signaling pathways (Condorelli et al., 2000). A pre-incubation with 200 ng/ml of the anti-TNFRI antibody, 1 h before exposure of retinal cells to 10 ng/ml of TNF-α for 48 h, was sufficient to prevent the decrease in cell viability triggered by TNF-α (data not shown). To study whether TNFRI could be mediating the decline in cell viability observed under high glucose conditions, incubation with 200 ng/ml of the antibody was performed 2 days after exposure of retinal cells to HG, prior to the HG-induced decline in cell viability. This time point was chosen taking into account the results obtained by MTT described above (Fig. 3A). As seen in Fig. 6B, blockade of TNFRI prevented the decrease in cell viability induced by HG. Cells exposed to HG alone show a relative cell viability of only 89.2 ± 1.2% of control while HG + anti-TNFRI show a significantly higher viability of 102.9 ± 1.3% of control (p < 0.001).

As mentioned above, the effect of high glucose exposure for long periods leads to a clear increase in apoptosis in retinal cell cultures. Therefore, in order to evaluate if apoptosis was also mediated by TNFRI, we have blocked this receptor with the anti-TNFRI antibody and performed the TUNEL assay. Incubation with anti-TNFRI decreased the number of apoptotic nuclei found in HG by about 40%, from 18.3 ± 1.1 TUNEL positive cells/field observed in retinal cells cultured in HG to 13.1 ± 1.1 TUNEL positive cells/field in HG incubated with the TNFRI antagonist (p < 0.01), as seen Fig. 6C, whereas TNFR1 inhibition did not alter the number of TUNEL+ -cells in control or mannitol conditions. This indicates that the HG-induced cell death is occurring through a TNFRI dependent mechanism. To further confirm this observation, the extent of caspase-3 activation was evaluated in control and HG cultured cells treated with anti-TNFRI. The results in Fig. 7 show that by inhibiting TNFR1 a reduction of 40% in the number of cells positive for activated caspase-3 is achieved (26.1 ± 3.6 in HG alone to 18.2 ± 2.5 positive cells/field in HG + anti-TNFRI; p < 0.01). Thus, it can be concluded that blocking TNFR1 prevents caspase-3 activation in HG cultured retinal cells and in this way promotes cell survival.

Discussion

Several reports have already established inflammatory mechanisms as crucial for the onset of diabetic retinopathy (Gaucher et al., 2007; Ibrahim et al., 2011; Krady et al., 2005) and in the early events of retinal neuronal apoptosis in diabetes (Asnaghi et al., 2003; Barber et al., 1998). Moreover, convincing evidence has been gathered casting TNF-α as a major player in retinal cell death (Behl et al., 2008; Joussen et al., 2009) and also in inflammatory processes in DR, such as leukocyte recruitment (Gustavsson et al., 2010; Joussen et al., 2002; Limb et al., 1996) and increased vascular permeability (Aveleira et al., 2010). In the present study, we demonstrate that blockade of TNFRI, a receptor that is highly expressed in synaptic terminals in retinal cell cultures, prevents the apoptotic cell death caused by elevated glucose concentration, a condition used to mimic hyperglycaemia.

TNF-α activates TNFR1 and TNFR2 and the signaling cascades following cytokine-cytokine receptor interaction for both receptors have been extensively described (Bradley and Pober, 2001; Heller and

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Fig. 6. Blockade of TNFR1 prevents retinal neural cell apoptosis induced by high glucose. (A) Representative images showing TUNEL staining (green) and nuclei staining with Hoechst (blue). Scale bar corresponds to 50 μm. (B) Anti-TNFRI prevents the decrease in MTT reduction in retinal cell cultures exposed to HG (30 mM) for 7 days. Data represent means ± SEM of 5–7 independent experiments performed in triplicate. (C) Blocking of TNFR1 prevents the increase in apoptosis in retinal neural cells induced by exposure to HG, as observed with the TUNEL assay together with nuclei staining (Hoechst) – only nuclei positive for both stainings were considered as cells undergoing apoptosis. Data are presented as number of TUNEL positive cells per field and represent means ± SEM of 3–4 independent experiments; **p < 0.01 compared to control; ***p < 0.01 compared to HG alone (two-way ANOVA followed by Bonferroni’s post test).
TNFR1 is considered the receptor most responsible for inducing cell death, by recruiting several adaptor protein through its intracellular Death Domain (Locksley et al., 2001; Tartaglia et al., 1993), while studies have shown TNFR2 to play mostly a neuroprotective and anti-inflammatory role in TNF signaling (Fontaine et al., 2002; Veroni et al., 2010). Still, notwithstanding its contribution for pro-survival signals, TNFR2 can also induce cell death indirectly, ultimately requiring TNFR1-mediated activation of apoptotic signaling pathways (Fotin-Mleczek et al., 2002).

Having such a determinant function in inflammation and cell death, TNFR1 was our main focus. Data obtained from immunofluorescence and colocalization studies show that in retinal cell cultures, TNFR1 is mainly present in neurons, with a particularly strong expression in neuronal synapses. TNFR1 has been shown to be involved in chemically induced apoptosis of hippocampal neurons in mice (Harry et al., 2008) and also to mediate the neurotoxic effects of microglia-derived TNF-α (Taylor et al., 2005; Yang et al., 2002). This suggests a considerable vulnerability of retinal neurons to increased levels of TNF-α in the extracellular milieu.

Using retinal neural cell cultures exposed to a high concentration of glucose, we investigated whether hyperglycaemia could affect the TNF signaling system. Despite showing no sign of altering TNFRs expression at the time point analyzed, high glucose still had an effect on the protein levels of TNFR1 in cultured retinal cells, resulting in a noticeable reduction in the amount of this receptor. The mechanism of “ectodomain shedding” is known to be mediated by, among other enzymes, TACE/ADAM17, a metalloproteinase discovered for its capacity to convert the transmembrane 26 kDa TNF-α into its 17 kDa soluble form (Black et al., 1997; Moss et al., 1997). This receptor shedding mechanism has been interpreted as a means of regulating TNF signaling (Mohler et al., 1993; Porteu and Nathan, 1990; Xanthoulea et al., 2004) and is also known to occur in TNFR1 and TNFR2 (Solomon et al., 1999).

Thus, given the robust decrease in TNFR1 observed in retinal cells cultured in HG, TNFR1 shedding by TACE could also be increased in the same conditions. However, we have found that both in terms of TACE protein levels and enzymatic activity, there were no differences between the three experimental conditions. Moreover, the levels of sTNFR1 in the culture media of retinal cells were decreased in comparison to control, at day 7 of exposure to high glucose, similar to the levels of the membrane anchored receptor. Thus, no evidence was found accounting the mechanism of receptor shedding for the decrease in TNFR1 levels. A genetic mutation within the TNFR1 extracellular region found in patients with TNF associated periodic syndrome (TRAPS), a chronic inflammatory disorder, leads to a decrease in the concentration of sTNFR1 in the serum (McDermott et al., 1999). Moreover, as reported by Waetzig et al. (2005), sTNFR1 can take part in immune homeostasis, limiting inflammation by inducing apoptosis in monocytes bearing transmembrane TNF-α, via reverse TNF signaling. This suggests that a reduction in sTNFR1 concentration, as observed here, might imbalance the pro-inflammatory and anti-inflammatory molecular signals toward a state of enhanced inflammation.

The required internalization of TNFR1 upon binding of TNF to start its cell death signaling pathway — through interaction with TRADD, FADD, caspase-8 and lastly caspase-3 — has been well established (Schneider-Brachert et al., 2004). After receptor internalization, TNFR1 containing vesicles fuse with late endosomes and end up accumulating lysosomal proteins such as CTSD and LAMP-1. One of the first studies evidencing this phenomenon, by Mosselmans et al. (1988), demonstrates that the fate of TNFRs after TNF binding results in the formation of secondary lysosomes, leading up to protein degradation. These observations might help explain the contrasting results obtained in the current study, where despite strong evidence for the involvement of TNFR1 in the observed high glucose-induced cell death, the levels of this receptor are found to be lower after exposure to high glucose. In this view, a decrease in the receptor’s protein levels could be a direct result of the pathophysiological activity of TNFR1. In addition, cells expressing TNFR1 might correspond to the population undergoing most extensively apoptosis and therefore, with these cells’ demise, so follows a decline in the total content of the receptor.

As previously described (Kusner et al., 2004), a decrease in cell viability appears to be an early event induced by high glucose/hyperglycaemia on Müller cells. Similarly, as shown in the present study, a significant decrease in retinal cell viability starts nearly 3 days after exposure to HG and, as the exposure period increased, the drop in retinal cell viability became more pronounced. At the onset of this event there was an increase in TNF-α expression, supporting a role for this pro-inflammatory cytokine in the HG-induced retinal cell death. Moreover, the reduction in cell viability was followed by a marked increase in apoptosis, observed at the longest exposure time.

TNFR1 induced cell death has been found to require caspase activation and the effector caspase-3 readily undergoes activation by caspase-8 upon TNF and FasL stimulation (Fotin-Mleczek et al., 2002). Therefore, we evaluated whether HG exposure affected the levels of active caspase-3 in our retinal cell cultures. In effect, the presence of cleaved caspase-3 was more pronounced in cells exposed to HG, further supporting the involvement of TNF-α in the neurodegeneration resulting from HG. It seems thus tempting to conclude that TNF-α, of which expression was increased early after high glucose incubation, is inducing retinal cell apoptosis in a caspase-dependent TNFR1-mediated manner. As reported in several models of peripheral nervous system development, TNFR1 is responsible for mediating the TNF-α-induced neuronal cell death (Barker et al., 2001) or, leastways, for priming peripheral neurons for apoptosis (Sedel et al., 2004). However, our current data is insufficient to exclude a likely contribution of TNF-α in triggering and/or exacerbating an inflammatory response. Given the presence of microglia in the cultures used in this study, TNF-α signaling through TNFR1 can further activate these cells (Kuno et al., 2005), taking part in a positive feedback loop, stimulating the release of gluta
tate (Takeuchi et al., 2006) and inflammatory mediators, thus increasing inflammation.

While many studies have accounted ganglion cell death for the neurodegeneration found in DR (Martin et al., 2004), other reports revealed a prominent role of photoreceptor apoptosis and outer nuclear layer histological alterations in diabetic patients (Cho et al., 2000) and experimental diabetes (Alvarez et al., 2010; Park et al., 2003). Regarding this question, in our model the increase in apoptosis was found to result from a prevalent degeneration of photoreceptor neurons, specifically rod photoreceptors, and rod bipolar cells. Despite the discrepancy among reports, both mechanisms, ganglion cell death and rod/cone degeneration, are unlikely to be independent events. Given the observed process of microglia invasion of the outer plexiform layer in DR (Ibrahim et al., 2011), our results contribute to elucidate, at least, this side of the retinal neurodegeneration found in diabetic
retinas. Moreover, the degeneration of retinal bipolar cells could account for the alterations in the electroretinogram, manifest as a reduction in oscillatory potential (OP) activity, resulting from diabetes (Tzekov and Arden, 1999).

Lastly, our results show that blocking TNF-R1 is sufficient to prevent retinal neural cell death induced by high glucose, revealing its involvement in the pathogenesis of DR, particularly in neural cell dysfunction/death, and opening ways for potential therapeutic approaches. Advances in the last decade have led to the introduction of several new drugs that act as blockers of TNF-α. This diverse class of drugs, belonging to the broader group referred as “biologics”, include full-length bivalent monoclonal anti-TNF-α antibodies (e.g. infliximab, adalimumab and golimumab) and TNFR-Fc fusion proteins (etanercept), among others. Despite the varied constructs employed in the synthesis of such molecules, all share the same pharmacological strategy: competing with endogenous receptors for the limited amount of available cytokine, thus impeding TNF-α signaling altogether. Much interest has been cast in the usage of these new drugs in diseases characterized by chronic inflammation, such as rheumatoid arthritis (RA) and Chron’s disease (CD), to name a few. Reports of successful treatments of RA with anti-TNF drugs date from as far as from 1994 (Elliott et al., 1994; Maini et al., 1999) and since then many other ailments have been added for potential therapeutic (reviewed in Tracey et al., 2008). In recent years, given the currently established contribution of TNF-α to the progression of DR, several studies have focused on the benefits such TNF antagonist-treatments might bring in countering the retinal vascular dysfunction found in diabetes (Behl et al., 2008; Joussen et al., 2009; Sifakis et al., 2005). The results presented in this work suggest that employing such drugs in the treatment and prevention of DR might go beyond the beneficial effects at the retinal microvasculature, protecting also the neural retina. Nonetheless, despite the positive prospects laid out by the above mentioned reports, as first proposed by Fontaine et al. (2002) the role of TNFR2 in retinal cell survival might be relevant in DR, as shown for retinal ischemia. Thus, strategies aimed at inhibiting the activity of TNFR1 while keeping TNF-α signaling through TNFR2 intact may end up turning to be of great value. Providing further support to the view that the pathogenic effects of TNF-α in the retina are mediated mainly by TNFR1, Kociok et al. (2006) have demonstrated the role this receptor plays in the pathological neovascularization, in a model of oxygen-induced retinopathy, through stimulation of VEGF expression. Biologic drugs targeted at receptors responsible for carrying out the cytokine’s noxious signals have also been developed. Tocilizumab, a monoclonal antibody targeted against the IL-6 receptor, has already been assessed for use in patients with RA, and the therapeutic potential of selective TNFR1 antagonists in diseases with an inflammatory background has started to be explored (Kontermann et al., 2008; Shibata et al., 2008). This strategy opens the possibility of interfering with the signaling mediated by one receptor, which in pathologies involving TNF-α can be advantageous since two distinct pathways with unique effects are at play: cell death and cell survival.

Conclusion

In this work we demonstrate that TNFR1 plays a central role in the high glucose-induced cell death in retinal cells and also present evidence supporting the antagonism of this receptor as a valuable therapeutic approach for prevention of the early cell death observed in diabetic retinopathy.

Experimental methods

Materials

Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD, USA) and trypsin (USP grade) was from Invitrogen Corporation (Carlsbad, CA, USA). The anti-TNF-R1, anti-TNF-R2, anti-Rhodopsin, anti-Calretinin and anti-TACE antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies selective for the cleaved caspase-3 (Asp175) and PKCα were purchased from Cell Signalling Technology (Beverly, MA). Hoechst 33342 and the secondary fluorescent antibodies, labelled with Alexa Fluor 488 or 594, were acquired from Invitrogen-Molecular Probes (Leiden, The Netherlands). The recombinant rat TNF-α, with specific activity of 5 × 10⁴ U/mg, was acquired from PeproTech (PeproTech EC, UK). Polyvinylidene difluoride (PVDF) membranes, the alkaline phosphatase-linked secondary antibodies and the enhanced chemiluminescence (ECL) reagent were purchased from GE Healthcare (Buckinghamshire, UK). Rat tumor necrosis factor-α and rat soluble tumor necrosis factor receptor 1 Quantikine Immunoassays were acquired from R&D Systems (Minneapolis, MN, USA). The TUNEL-FITC kit (In Situ Cell Death Detection Kit, Fluorescein) was purchased from Roche Applied Science (Mannheim, Germany) and the Sensolylte™ 520 TACE (α-Secretase) Activity Assay Fluorescent Kit from AnaSpec Inc (Fremont, CA, USA). Primers used for qRT-PCR were synthesized by MWG Biotech (Ebersberg, Germany). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck KGaA (Darmstadt, Germany).

Retinal cell culture

Cell cultures were obtained from 3 to 5 days old newborn Wistar rats, as previously described (Santiago et al., 2006). Briefly, neonatal rats were sacrificed by decapitation, the eyes were removed and the retinas dissected in Ca²⁺-and Mg²⁺-free Hank’s balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH₂PO₄, 0.34 Na₂HPO₄, 4 NaHCO₃, 5 glucose; pH 7.4) under sterile conditions, using a light microscope, followed by a digestion with 0.05% trypsin (w/v) for 15 min at 37 °C. After dissociation, cells were plated at a density of 2.0 × 10⁶ cells/cm² on glass coverslips or in 35 mm petri dishes, coated with poly-D-lysine (0.1 mg/ml), and were maintained in Eagle’s minimum essential medium (5 mM glucose) supplemented with 25 mM HEPES, 26 mM NaHCO₃, 10% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml), at 37 °C in a humidified atmosphere with 5% CO₂. After two days, the culture medium was supplemented with 25 mM d-glucose, reaching a final concentration of 30 mM glucose (HG cells) and cultured for further 2–7 days, while in the control group glucose concentration was maintained at 5 mM. d-mannitol (25 mM) was used as an osmotic control.

Immunocytochemistry

Cells cultured in glass coverslips were fixed in paraformaldehyde solution (4% paraformaldehyde, 4% sucrose in phosphate-buffered saline, PBS), for 20 min at room temperature, and then permeabilized with 1% Triton X-100 in PBS for 5 min. Fixed cells were then blocked for 1 h with 3% BSA plus 0.2% Tween 20 in PBS, followed by an incubation with the primary antibodies (rabbit anti-TNF-R1 1:200, rabbit anti-TNF-R2 1:100, rabbit anti-cleaved caspase-3 1:1600, mouse anti-synaptophysin, 1:400) overnight at 4 °C. After washing, cells were incubated for 1 h, at room temperature, with the secondary antibodies (AlexaTM 488 anti-mouse IgG, 1:200, or AlexaTM 594 anti-rat IgG 1:200). Cell nuclei were stained using Hoechst 33342 (1 ug/ml, 5 min). Coverslips were mounted and images taken in a Carl Zeiss Laser Scannig Confocal Microscope LSM 510.

Image analysis

Confocal optical sections were used for colocalization analysis. Acquisitions were made using the Carl Zeiss Laser Scanning System LSM 510 software and posterior analysis was carried out in ImageJ. Colocalization was evaluated based on the Pearson’s correlation coefficient
genes. The amplification mRNA level ratios calculated using the altered Pfaf
(TATA box binding protein, TBP; Peptidylprolyl isomerase A, Ppia; hypoxanthine guanine phosphoribosyl transferase 1, Hprt1) and the
30 s step at 72 °C for elongation. Data from the target genes were
by 30 s at the annealing temperature optimal for each primer and lastly
step protocol, consisting of a 10 s denaturation step at 95 °C, followed
tomated gel-electrophoresis system (Bio-Rad). For cDNA synthesis, 1
water and its quality and integrity was assessed using the Experion au-

Retinal cells, cultured in 35 mm petri dishes, were washed and 
ysed at 4 °C with RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 
 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, with protease and phosphatase inhibitor cocktails). The lysates were first sonicated, then centrifuged at 13 000 g for 10 min at 4 °C and the supernatants were collected. The protein concentration was determined by the BCA method and afterwards denatured by addition of 6× concentrated loading buffer (0.5 M Tris, 30% glyceral, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and by heating the samples for 5 min at 95 °C. Proteins were separated in a 10% sodium dodecyl sulphate–polyacrylamide gel by electrophoresis (SDS-PAGE) and transferred electrophoretically to PVDF membranes. Membranes were blocked for 1 h at room temperature, in Tris-buffered saline (in mM: 137 NaCl, 20 Tris–HCl; pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk. The membranes were incubated with the antibodies (rabbit anti-TNFRI 1:500; rabbit anti-TNFRII 1:500, goat anti-TACE 1:500, mouse anti-actin 1:20000), overnight at 4 °C under continuous shaking. After washing in TBS-T, the membranes were incubated for 1 h, at room temperature, under continuous shaking, with the secondary antibodies (anti-rabbit IgG 1:20000, anti-mouse 1:20000 or anti-goat IgG 1:5000). Protein immuno-reactive bands were visualized using the ECF system, on a gel im-
ger (Versa Doc Imaging System; Bio-Rad), and digital quantification was performed using Quantity One software (Bio-Rad).

Quantitative real time RT-PCR assay

Total RNA was isolated from retinal cells cultured in 35 mm petri dishes, using the RNeasy Mini Kit from Qiagen according to the manu-
ufacturer’s instructions. Briefly, cells were lysed and the extracts kept at 
-80 °C until RNA isolation. Isolated RNA was eluted in Rnase-free water and its quality and integrity was assessed using the Experion au-
tomated gel-electrophoresis system (Bio-Rad). For cDNA synthesis, 1 µg 
of RNA was used and reverse transcription carried out using the iScript-
cDNA synthesis (Bio-Rad). Synthesized cDNA, diluted from 1:10 to 
1:100, was used for the amplification of the desired genes using a 3 
step protocol, consisting of a 10 s denaturation step at 95 °C, followed 
by 30 s at the annealing temperature optimal for each primer and lastly 
a 30 s step at 72 °C for elongation. Data from the target genes were 
normalized using the expression of three stable reference genes (TATA 
box binding protein, TBP; Peptidylprolyl isomerase A, Ppia; hy-
pyoxanthe amine phosphoribosyl transferase 1, Hprt1) and the 
miRNA level ratios calculated using the altered Pfaf model described 
in Hellemans et al. (2007), for normalizations with multiple reference 
genes. The amplified genes and their respective primers are as follows: 
TNF-α, forward 5′-ACA GCA CCT CCA GAC C-3′, reverse 5′-AGT 
TCC ACA TCT CGG ATG ATG-3′; TNFR1 forward 5′-TCT CCT 
CTT CTT TCT GC-3′ reverse TCT TGA GAA ACT CAT CAC TGT 
AT TGG A-3′; TNFR2 forward 5′-GGT GGC TTC CCT CTT CCA 
AT-3′ reverse 5′-GTT CGC CAG TCC TAAA CAT CAG-3′; 
TBP forward 5′-CTA ACC ACA GCA CCA TCT-3′ reverse 5′- 
TCA CAG ACA ATC ACG-3′; Ppia forward 5′-TGT GGG AGG GTG 
AAA GAA GCC-3′ Reverse 5′-ACA GCA CCT ATG TTA TGA GGG-3′; 
Hprt1 forward 5′-CCT TCA TCT CAT TAA AAG GCA 
TCC-3′. PCR products for each gene were submitted 
cluding the generated amplicons and thus the specificity of 
selected primers.

TACE activity assay

For measuring the activity of the tumor necrosis factor-α converting 
(TACE) a fluorimetric assay, Sensolyte 520 TACE Activity 
Assay, was used. This assay relies on a FRET peptide, derived from a 
sequence surrounding the cleavage site of TACE, containing a fluoro-
phore (5-carboxyfluorescein, 5-FAM) and a quencher (QXL 520). In 
the intact FRET peptide the fluorescence of 5-FAM is quenched by 
QXL 520 but when the active TACE enzyme cleaves this substrate it 
results in the recovery of 5-FAM fluorescence. The standard protocol 
provided by the manufacturer was followed. Briefly, retinal cell cul-
tures were lysed with 50 µl of the supplied Assay Buffer supplemented 
with 0.1% Triton-X and centrifuged at 2500 rpm for 10 min, at 
4 °C. Protein content was measured as described above and samples 
were stored at -80 °C until use. For the assay, 10 µg of protein were 
used. 5-FAM fluorescence was monitored at excitation/emission 
velocities of 490 nm/520 nm, respectively, for 60 min at 37 °C.

sTNFR1 enzyme linked immunosorbent assay

Soluble tumor necrosis factor receptor 1 levels were measured in 
cell culture media using the respective enzyme-linked immunosorbent 
assay (ELISA) and according to the protocol provided by the manufac-
turer. At the end of incubation, the culture medium from cells in control, high glucose and mannitol conditions was collected, cleared by cen-
trifugation and stored at -80 °C until use. A minimum of 100 µl of 
media was loaded on the ELISA and the colorimetric quantification 
was carried out at 450 nm (with correction at 570 nm).

MTT cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to assess cell viability in retinal 
cells. MTT is taken up by living cells and reduced to an insoluble pur-
ple formazan salt by cellular dehydrogenases (Mosmann, 1983). Briefly, 
cells were washed with Krebs medium (in mM: 132 NaCl, 4 KCl, 1.4 
MgCl2, 1.4 CaCl2, 6 glucose, 10 HEPES; pH 7.4) and then incubated with 
the MTT (0.5 mg/ml) dye in Krebs for 1 h at 37 °C and 5% CO2. 

TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end 
labeling (TUNEL) assay was used for detecting cell apoptosis. The 
standard protocol provided by the supplier was followed. Retinal cells, fixed 
and permeabilized as previously described, were incubated at 37 °C 
for 1 h with the terminal deoxynucleotidyl transferase enzyme and 
fluorescein-labelled nucleotides, after which cells were rinsed with 
PBS followed by Hoechst staining. Coverslips were mounted using a 
Prolong Antifade Kit and visualized using a fluorescence microscope 
(Zeiss Axioscope 2 Plus) coupled to a digital camera (Axiocam HRC).

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was de-
termined using one-way ANOVA, as specified in the figure legends, 
followed by Bonferroni’s post hoc test, unless otherwise stated.

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