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Somatostatin protects human retinal pericytes from inflammation mediated by microglia

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Abstract

Diabetic retinopathy (DR) is usually considered a microvascular disease. However, involvement of the neuroretina in the early stages of DR has recently gained major credit. Inflammatory processes, leading to glial activation and neuronal apoptosis, develop early in the retina of diabetic subjects. Pericytes constitute a link between the vascular and the neural retina, play a central role in blood-retinal barrier maintenance, and may influence neuroinflammation. Somatostatin (SST) is a potent neuroprotective factor, which is downregulated during early DR. In this paper, we have investigated the effects of the inflammatory signals triggered by the activation of microglia on inflammation and apoptosis/survival pathways in pericytes. Microglia cells (Bv-2) were stimulated with lipopolysaccharide (LPS) and/or SST. Human retinal pericytes (HRP) were exposed to conditioned media (CM) collected from Bv-2 cells in physiological conditions and in the settings described above. A panel of inflammation, apoptosis and survival mediators was analyzed. HRP treated with LPS-CM showed a significant increase of pro-inflammatory (iNos and TNFa) and proapoptotic mediators (FasL, active caspase-8, tBid and Bax), and a concomitant decrease in pro-survival factors (BclxL and pAkt). SST added to LPS was able to counteract these effects in all conditions. In conclusion, SST is able to modulate apoptosis/survival pathways in HRP during microglia-mediated inflammation. These results demonstrate a crosstalk between microglia and retinal pericytes, evidencing a possible defensive role of microglia in the early phases of DR.

Keywords: diabetic retinopathy; somatostatin; pericyte; microglia; inflammation; apoptosis

Abbreviations: CM, conditioned medium; DME, diabetic macula edema; DR, diabetic retinopathy; FasL, Fas ligand; FBS, fetal bovine serum; HRP, human retinal pericytes; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; PDGF-B, Platelet-derived Growth Factor B; PDR, proliferative diabetic retinopathy, SST, somatostatin; TNFα, Tumor Necrosis Factor-α

1. Introduction

Diabetic retinopathy (DR), a sight-threatening complication of diabetes, has long been considered a microvascular disease. Two early events in its pathogenesis, disappearance of pericytes and thickening of the basement membrane, which take place inside the capillaries, may in fact lead to loss of control on endothelial proliferation and, consequently, abnormal angiogenesis (Gerhardt and Betsholtz, 2003; Armulik et al., 2005). In the latest years, however, the involvement of the neuronal part of the retina in the early stages of DR has gained major credit (Barber, 2003; Antonetti et al., 2005; Hernández et al., 2013).

Diabetes-induced microvascular and neural alterations share several features, such as oxidative stress (Nishikawa et al., 2000; Silva et al., 2009), increased production of advanced glycation end products (Nishikawa et al., 2000; Berner et al., 2012), and activation of the renin-angiotensin system (Marin Garcia and Marin-Castaño, 2014). Moreover, pericytes are coming more and more in the focus of neuroscientists because they constitute a link between the vascular and the neural sides of the retina and play a central role in blood-brain/blood-retinal barrier maintenance (Navarro et al., 2016; Sweeney et al., 2016). In fact, pericytes control endothelial cell–mediated leukocyte adhesion and transmigration into the central nervous system (Olson and Soriano, 2011), while *in vitro* studies have shown that pericytes may influence neuroinflammation (Jansson et al., 2014).

Inflammatory processes, hallmarks of retinal neurodegeneration and leading to glial activation and neuronal apoptosis, have been found in the retina of diabetic subjects in the early phases of DR (Carrasco et al., 2005; Hernández et al., 2013). During DR, the inflammatory process is mediated by resident immune cells, which, depending on their polarization, can trigger pro- (M1) or anti-inflammatory (M2) actions (Linch, 2009). Microglial cells are considered as key-players in the inflammatory processes, as they take part in the defensive immune system by releasing neuroprotective and anti-inflammatory factors (Zabel and Kirsch, 2013). However, during DR and in response to a constant pro-inflammatory insult, retinal microglial cells switch their polarization from M2 to M1 (Arroba et al., 2016a), with consequent release of deleterious cytokines related to retinal neurodegeneration (Linch, 2009, Zabel and Kirsch, 2013).

Platelet-derived growth factor B (PDGF-B), secreted by the endothelium and its receptor PDGFR- β , expressed by pericytes, are essential molecules for the proliferation, migration and recruitment of pericytes to the vasculature (Hammes et al, 2002; Beltramo and Porta, 2013). Decreased PDGFR- β signaling, as a consequence of hyperglycemia, results in pericyte apoptosis in animal models of DR (Geraldes et al., 2009), but also mediates pro-inflammatory responses by promoting endothelial release of monocyte chemoattractant protein-1 (MCP-1), nitric oxide (NO), interleukins IL-1, IL-6, IL-12, and tumor necrosis factor- α (TNF α) (Olson and Soriano, 2011).

Somatostatin (SST), an important neuroprotective factor synthesized inside the retina, has anti-angiogenic properties (Simó et al., 2006) and is able to modulate several ion/water transport systems (Cervia et al. 2008); thus, it may prevent proliferative DR (PDR) and diabetic macular edema (DME). Downregulation of SST in the early stages of DR has been described (Carrasco et al., 2005), while the intravitreal injection of SST and SST analogues (Kiagiadaki et al., 2010), as well as topical administration in eye drops, prevent diabetes-induced retinal neurodegeneration (Hernández et al., 2013). We have recently reported that SST

reduces high glucose-induced caspase-8 activity in photoreceptor cells and retinal explants (Arroba et al., 2016b), while it has no direct effects on human retinal pericytes (Beltramo et al., 2016).

In this study, we aimed at investigating the effects of inflammation mediated by the microglia on pericyte apoptosis/survival pathways and also evaluate if SST could exert a protective function.

2. Materials and methods

2.1 Cell models

Mouse microglia Bv-2 cell line was provided by Dr. M.L. Nieto (CSIC, Spain). Bv-2 cells were cultured in RPMI supplemented with 10% FBS (Sigma Aldrich, Saint Louis, MO, USA). Cells were grown up to 70% confluence, and then stimulated for 24 h in serum-free medium with 200 ng/ml lipopolysaccharide (LPS, Sigma Aldrich) to induce inflammation (M1 response), or 10⁻⁷ M somatostatin (SST, a generous gift by BCN Peptides, Barcelona, Spain), or with LPS and SST together. Conditioned media (CM) were collected and centrifuged to remove cell debris, and supernatants added to pericyte cultures (see below). Bv-2 cells were also exposed to high glucose concentrations (HG, 25 mM) with or without the addition of SST.

Human retinal pericytes (HRP) were stabilized in our laboratory (Berrone et al, 2009). They were usually grown in DMEM + 10% FBS (Sigma Aldrich). For CM experiments, HRP were cultured in 50% (DMEM + 10% FBS) and 50% CM from Bv-2 cells. Media were changed every 48 hrs and cultures maintained for 8 days.

2.2 Quantitative Real Time PCR (qRT-PCR)

The mRNA expression on TNFα, IL-1β and IL-6 in Bv-2 cells after exposure to LPS/HG/SST was evaluated by qRT-PCR. Total RNA was extracted by HighPure RNA Isolation kit (Roche). After spectrophotometric quantification (Nanodrop ND-1000), 200 ng RNA were reverse-transcribed using miScript Reverse Transcription Kit (Qiagen). qRT-PCR was performed by 48-well StepOne Real Time System (Applied Biosystems) using a miScript SYBR Green PCR Kit (Qiagen). Commercially-available primers (ThermoFisher Scientific, Waltham, MA, USA) were used. RNA expression was normalized against the small nuclear RNA RNU6B.

2.3 Cell survival parameters

HRP proliferation was measured as DNA synthesis (*Cell Proliferation ELISA BrdU kit*, Roche Diagnostics, Basel, Switzerland) and apoptosis as DNA fragmentation (*Cell Death Detection ELISAPLUS kit*, Roche). Results were checked by a fluorescent/chemiluminescent assay, which measures viability, cytotoxicity and apoptosis in the same well (*ApoTox-Glo™ Triplex Assay, Promega Corporation, Madison, WI, USA*). All procedures were carried out according to manufacturers' instructions.

2.4 Protein extraction

To extract total proteins, HRP were lysed using *M-PER Mammalian Protein extraction reagent* (ThermoFisher) added with 10 µl/ml *protease inhibitor cocktail kit* (ThermoFisher). Extracts were kept ice-

cold and cleared by centrifugation at 20,000 g for 15 min at 4°C. The supernatant was aliquoted and stored at -80° C.

Other sets of cells were processed for extraction of separate cytoplasmic and nuclear fractions *using* NE- PER^{TM} *Nuclear and Cytoplasmic Extraction Reagents* (ThermoFisher), accordingly to the manufacturer's instructions. The two cellular fractions were separated by addition of different ice-cold extraction buffers to the cell pellet and stored at -80° C. Protein concentration was determined using the Bradford method.

2.5 Western blot analysis

Protein (30 µg) were loaded on sodium dodecyl sulphate polyacrylamide gel and separated by electrophoresis. Gels were transferred to Immobilon membranes (Merck-Millipore, Billerica, MA, USA). Membranes were blocked using 5% non-fat dried milk in 10 mmol/l Tris-HCl and 150 mmol/l NaCl pH 7.5 and incubated overnight with relevant antibodies in 0.05% Tween-20, 10 mmol/l Tris-HCl, and 150 mmol/l NaCl pH 7.5.

All primary antibodies were used at a 1:1000 dilution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting protocol (Merck-Millipore). Antibodies used were: anti-FasL (AB-16982) and anti-IL6 (MABF41) (Merck-Millipore); anti-BclxL (551269, BD Biosciences PharMingen, San Diego, CA); anti-Bid (AF860, R&D Systems, Minneapolis, MN, USA); anti-cleaved (Asp175) caspase-3 antibody (9661), anti-p53 (9282S), anti-IL-1 β (3A6) (12242) and anti-histone H3 (D1H2) (4499) (Cell Signaling, Beverly, MA, USA); anti-iNOS (sc-650), anti-phospho-Akt1/2/3(Ser473) (sc-7985-R), anti-Akt1/2/3 (sc-8312), anti-caspase-8 (sc-7890), anti-PCNA (sc-56) and anti-Bax (sc-493, Santa Cruz Biotechnology, Palo Alto, CA, USA); anti-TNF α (ab9635) (Abcam, Cambridge, UK); anti- α -tubulin (T0198) and anti- β -Actin (A5316, Sigma-Aldrich).

The relative signal strength was quantified by densitometric analysis (ImageJ software, NIH, USA), and values normalized against α -tubulin. Regarding activated proteins, results were expressed as the ratio of active to pro/inactive forms: active caspase-8/pro-caspase-8, truncated Bid (tBid)/Bid, phosphorylated Akt (pAKT)/Akt. In experiments aimed at verifying active caspase-8 nuclear translocation, β -actin and histone H3 were used as cytoplasmic and nuclear loading controls, respectively.

2.6 Immunofluorescence staining

Pericytes were fixed in ice-cold methanol for 5 min at -20°C, dried at RT for 15 min and re-hydrated in PBS for 15 min. Non-specific binding sites were blocked in PBS plus 0.2% BSA (blocking solution) for 1h at RT. Cells were then incubated overnight at 4°C with 1 µg/ml anti-cleaved-caspase-8 (ab25901) or anti-cleaved caspase-3 antibodies (ab13847, Abcam). After washing 3 times with blocking solution, a FITC-conjugated goat anti-rabbit IgG (Sigma Aldrich), 1/1000 in blocking solution, was added for 1h at RT. DAPI was used to blue-stain the cell nuclei. Images were taken under a Leica DM 2000 microscope (Leica, Wetzlar, Germany), equipped with a Leica DFC 320 camera and Leica QWin Plus 2003 digital processing and analysis software.

2.7 Statistical analysis

As previously determined by a power analysis (SPSS software version 23.0, IBM), the minimum sample size that permitted to detect a 20% difference between the experimental groups with a 80% power and a

probability level of 0.05, two-tailed hypothesis (Student's *t*-test for paired data and/or Wilcoxon's Signed Ranks test) was N=5. Results are therefore expressed as mean \pm SD of 5 independent experiments, normalized against HRP cultured in physiological conditions (noCM).

3. Results

3.1 Release of pro-inflammatory cytokines by Bv-2 microglial cells following exposure to LPS and HG In order to evaluate if HG may induce an inflammatory response in microglial cells comparable to the one induced by LPS, Bv-2 were exposed to both LPS and HG, alone or with the addition of SST. As shown in **Fig. 1**, both stimuli induce an increase in mRNA expression of TNF α , IL-1 β and IL-6. These increases were comparable as regards TNF α and IL-6, while LPS induced a much higher increase in IL-1 β mRNA expression than HG (**Fig. 1 c-d**). SST was able to reduce the expression of pro-inflammatory molecules in all cases, when added to cells stimulated with LPS or HG.

3.2 Direct and microglia-mediated effects of SST and LPS on pericyte proliferation and apoptosis

We evaluated HRP proliferation and apoptosis following 8-day exposure to SST, LPS and SST+LPS to verify if these substances may have direct effects on pericytes. No differences in HRP proliferation, evaluated as DNA synthesis, and a slight, but not significant, increase in apoptosis (measured as DNA fragmentation) were found in HRP treated with LPS (data not shown).

Subsequently, we measured microglia-mediated effects on HRP proliferation and apoptosis by culturing HRP in CM from Bv-2 cells cultured in basal conditions (basal-CM), SST (SST-CM), LPS (LPS-CM) and SST+LPS-CM, as compared with HRP cultured in physiological conditions (noCM).

Results show that LPS-CM is able to decrease HRP proliferation (-11.4%, p<0.05 vs noCM) (**Fig. 2a**) and increase their apoptosis (+28.0%, p<0.05) (**Fig. 2b**), while addition of SST to LPS when culturing Bv-2 cells is able to normalize apoptosis.

3.3 Inflammatory markers

As regards pro-inflammatory markers, HRP treated with LPS-CM show increased expression of iNos and TNF α (**Fig. 3**). In particular, iNos expression was increased by 69.7% in comparison with both control without CM and basal-CM (p<0.05), while addition of SST alone to Bv-2 cells did not induce any change and SST added to LPS was able to counteract the LPS damaging effect (-67.2%, p<0.05) (**Fig. 3a**).

TNF α expression was +60.0% in HRP treated with LPS-CM (p<0.05 vs no CM and basal CM), while addition of SST to LPS decreased by 74.0% TNF α expression in comparison with noCM conditions (p<0.05 vs all conditions) (**Fig. 3b**). IL-6 and IL-1 β expression did not change significantly in any condition tested (data not shown).

3.4 Apoptosis mediators

Apoptotic and survival molecules known to be activated and/or decreased under these stress experimental conditions were examined. Regarding pro-apoptotic markers, we studied the expression of mediators involved in the apoptotic cascade in HRP in stress conditions (Beltramo et al., 2017): Fas ligand (FasL), Bid and its truncated active form (tBid), Bax, p53, pro/active caspase-8. We also checked the expression of caspase-3, which is activated by caspase-8 and considered one of the main executioners of apoptosis.

We observed significant increases of FasL, active caspase-8, and tBid protein expression in HRP treated with LPS-CM, and a normalization when HRP were treated with LPS + SST-CM (**Fig. 4**). In particular, FasL reached a 85% increase of in HRP treated with LPS-CM (p<0.005 vs noCM and basal-CM), while SST alone induced a 60% decrease of as compared with noCM (p<0.005) and the combination of the two completely normalized FasL overexpression due to LPS (p<0.05 LPS-CM) (**Fig. 4a**). Active/pro-caspase-8 ratio increased dramatically in LPS-CM (+122.6% in comparison with noCM and +95.1% vs basal-CM, p<0.05 vs both), while the addition of SST to LPS brought it back to -22.6% in respect to noCM (**Fig. 4b**). t-Bid/Bid registered a 76 % increase in LPS-CM (p<0.05 vs noCM and basal-CM), completely counteracted by the addition of SST (**Fig. 4c**). Bax and p53 were slightly, though not significantly, increased in LPS-CM, but the addition of SST to LPS in culturing BV-2 reduced their expression in HRP (**Fig. 4 d-e**). Finally, caspase-3 expression did not change in our experimental conditions.

3.5 Survival factors

As regards pro-survival factors, we examined the expression of phosphorylated Akt (pAkt Ser473)/Akt, BclxL, and proliferating cell nuclear antigen (PCNA) in HRP exposed to CM.

We found that LPS-CM induces a decrease of BclxL (-67%, p<0.05 vs noCM) and pAkt(ser473)/Akt (-37% vs SST, p<0.05), which is again counteracted by the addition of SST to LPS (p<0.05 vs LPS in both cases (**Fig. 5 a-b**), while PCNA expression did not change.

3.6 Caspase-8 nuclear translocation

Since no change in caspase-3 expression was found in our experimental setting, we checked the hypothesis that caspase-8 could act by a-direct translocation from the cytoplasm into the nucleus to induce DNA fragmentation. We measured active/pro-caspase-8 expression in cytoplasmic vs nuclear enriched fractions. As shown in **Fig. 6 a-d**, there was a 3-fold increase in the ratio active/pro-caspase-8 in the nuclear fraction of HRP cultured in LPS-CM (p<0.05 vs no CM and basal-CM), a decrease in SST-CM (-60% vs noCM and - 200% vs basal CM, p<0.05 vs both) and a normalization in SST+LPS-CM (**Fig. 6 b-d**), while no significant differences were found into the cytoplasmic fraction (**Fig. 6 a-c**). These data were confirmed by immunofluorescence staining (**Fig. 6 e-g**). Caspase-3 immunofluorescence is located inside the cytoplasm in all cases, and its expression was not affected by the treatments (**Fig. 6 e**). Immunolocalization of active caspase-8 into the nucleus is evident in HRP cultured in LPS-CM conditions, while addition of SST to LPS partially counteracts this effect (**Fig. 6 f-g**).

4. Discussion

In this study, we investigated the effects of inflammation mediated by the microglia on pericyte release of pro-inflammatory molecules, and apoptosis and survival pathways. We demonstrate that some inflammation markers and pro-apoptotic intermediates are increased in HRP cultured in CM obtained by exposing Bv-2 microglial cells to an inflammatory stimulus (LPS), concurrently with decreased expression of survival molecules, leading to an imbalance towards apoptosis. Importantly, the co-treatment SST-LPS during Bv-2 cell culture counteracts these effects, indicating that somatostatin may exert a protective function on HRP through microglial cells.

The major focus of our paper was to identify a cross-talk between microglia and pericytes mediated by the pro-inflammatory milieu associated to diabetes. Taking this into account and considering the difficulties in handling with human microglia, we have used Bv-2 murine microglial cells and immortalized human retinal pericytes. The pro-inflammatory cytokines analyzed in the present work show very similar sequences in humans and mice. Also, it has been reported that murine cytokines bind to human receptors and exert similar functions (Dower et al., 1986; Kuprash et al., 1999). Our results clearly demonstrate that the pro-inflammatory milieu generated by LPS-stimulated murine microglia is able to target apoptosis/survival pathways in human pericytes.

Pericytes play a central role in the development of DR since their early disappearance leads to the loss of control on endothelial proliferation, resulting in abnormal angiogenesis (Armulik et al., 2005). As a matter of fact, DR has long been considered a merely microvascular disease. Recently, however, it has been postulated a primary role for retinal neurodegeneration (Barber, 2003; Antonetti et al., 2005), caused mainly by pro-inflammatory processes, which develop early in the retina of diabetic subjects (Carrasco et al., 2005; Hernández et al., 2013). Pericytes, due to their localization, constitute a sort of bridge between the microvessels and the neuroretina.

LPS has been used as a pro-inflammatory stimulus, since we have previously reported that it is able to induce activation of Bv-2 microglial cells enhancing their M1 response (Arroba et al, 2016a). To better connect our results with the onset of DR, we have compared the effect of LPS with that of HG in Bv-2 cells and demonstrated that the inflammatory response induced by both stimuli is comparable, as well as the protective effect of SST.

In our experimental setting, LPS does not exert evident direct effects on HRP survival parameters, apart from a slight increase in their apoptosis. Controversial data have been reported in the literature on the potential damaging effects of LPS on pericytes (Maier & Pober, 2011; Ding et al, 2017). However, pericytes are quite a heterogeneous type of cells, and their response to various stimuli may vary depending upon their origin (Maier & Pober, 2011). In this regard, we have previously demonstrated that human and bovine retinal pericytes behave rather differently in their response to high glucose-induced damage (Beltramo et al, 2009).

On the other hand, HRP exposure to CM derived from microglia exposed to LPS decreases their proliferation and increases their apoptosis. This may be due to the release in the supernatant, by LPS-activated microglia, of different cytokines, such as TNF α and IL-1 β (Ding et al., 2017). Addition of SST to LPS in microglia cultures is able to neutralize this effect.

Pericyte capability of releasing pro-inflammatory molecules in response to stress stimuli has been described previously (Kovac et al., 2011; Navarro et al., 2016). This inflammatory response contributes to disruption of the blood retinal barrier and the consequent angiogenesis, thus playing a role in microvascular DR (Ogura et al., 2017). Accordingly, in this work, we show that HRP overexpress iNos and TNF α when exposed to CM obtained culturing microglia cells in the presence of LPS, while the secretion of IL-1 β and IL-6 is not changed. Since pericytes in the retina are located between neural tissue and microvessels, damaging stimuli derived from early neuroinflammation, through the pericyte filter, may affect the endothelium and contribute to microvascular damage.

Regarding the release of apoptotic factors in our model of crosstalk between microvascular and neuroretina, we investigated the expression of key-mediators of both the extrinsic and the intrinsic pathways, since we have recently demonstrated an involvement of both pathways in high glucose and hypoxia-mediated damage in HRP (Beltramo et al, 2017). We found significant increases of FasL, active capase-8 and t-Bid in HRP exposed to LPS-CM. FasL and active caspase-8 are classically involved in the apoptotic extrinsic pathway, that is characterized by the activation of transmembrane receptors belonging to the superfamily of TNF receptors, of which one of the best characterized is Fas (Elmore, 2007). The binding of FasL to Fas receptor activates a cascade of intracellular factors, leading to cleavage of procaspase-8 to active caspase-8 (Wajant, 2002). tBid and p53 have been described in both the intrinsic (mitochondrial) and extrinsic pathways of apoptosis and may act as mediators linking both pathways (Kiraz et al. 2016). A co-contributor of mitochondrial damage may be, in fact, caspase-8-mediated cleavage of Bid (Li et al. 1998; Esposti, 2002), while p53 may also be directly activated by caspase-8 (Kiraz et al., 2016). As Bax classically belongs to the intrinsic mitochondria-mediated pathway, our results demonstrate an involvement of both apoptotic pathways in the cross-talk between microglia and HRP, with caspase-8 playing a central role.

Examining the expression of pro-survival factors, we show decreased expression of BclxL and pAkt(ser473)/Akt in HRP exposed to LPS-CM. BclxL is an anti-apoptotic member of the Bcl family, while the phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays a crucial role in cell survival and growth during development, since activation of Akt by phosphorylation in Ser473 promotes cell survival and proliferation by inhibiting some of the pro-apoptotic Bcl-2 family members (Zhang et al., 2011).

Taken together, our data suggest an imbalance between pro-apoptotic and pro-survival factors in human retinal pericytes exposed to conditioned media from LPS-activated microglia.

Caspase-3 is activated by the initiator caspases (including caspase-8) and is generally considered the most important executioner caspase responsible for the activation of endonucleases, leading to DNA fragmentation, and proteases, degrading nuclear and cytoskeletal proteins (Elmore, 2007; Kiraz et al. 2016). Consistently with our previous findings (Beltramo et al., 2017), present results do not show any significant difference in caspase-3 expression in pericytes. However, we observed a translocation of caspase-8 from the cytoplasm into the nucleus following exposure of HRP to LPS-CM, in agreement with our and others' previous observations, demonstrating that active caspase-8 may bypass caspase-3 by directly acting as an executioner caspase, re-locating inside the nucleus and cleaving PARP-2, a member of the poly(ADP-ribose) polymerase family involved in DNA repair (Benchoua et al., 2002; Arroba et al., 2005; Beltramo et al., 2017).

SST, which is synthesized inside the retina and has neuroprotective and anti-angiogenic functions (Simó et al., 2006), has been shown to improve visual loss, prevent diabetes-induced retinal neurodegeneration, reduce caspase-8 activity and apoptosis in the retina of hyperglycemic rats (Hernández et al., 2013). In agreement with our work demonstrating that SST is able to reduce high glucose-induced caspase-8 activity in photoreceptor cells and retinal explants (Arroba et al., 2016b), in this study we show that addition of SST to LPS during microglia cell culture completely antagonizes inflammation-induced damage in pericytes, by reducing the expression of pro-inflammatory markers and counteracting the imbalance between apoptotic and survival intermediates.

In conclusion, pro- inflammatory stimuli mediated by the microglia are able to induce the release of inflammatory mediators by human retinal pericytes. Moreover, microglia-mediated inflammation induces pericyte apoptosis through the activation of pro-apoptotic and the inhibition of pro-survival molecules, thus leading to an imbalance between apoptotic and survival signalling pathways, with caspase-8 playing a primary and independent role. Taken together, all these phenomena strongly suggest the existence of a crosstalk between microglia and retinal pericytes and may contribute to the disruption of the blood-retinal barrier in early DR. SST is able to counteract most of these damaging effects, strengthening the hypothesis that this molecule may find a role in the prevention of DR.

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The Authors declare absence of commercial or propriety interest.

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Figures



Fig. 1. Expression of **inflammation markers** by Bv-2 microglial cells cultured in physiological conditions (basal), SST, LPS and LPS+SST (**a**, **c**, **e**), and in physiological conditions (basal), SST, HG and HG+SST (**b**, **d**, **f**). **a**, **b**) TNF α , **c**, **d**) IL-1 β , **e**, **f**) IL-6 mRNA expression. Mean of 5 experiments ± SD, *p <0.05 vs basal, £ p<0.05 vs SST, \$ p<0.05 vs LPS, # p<0.05 vs HG.



Fig. 2. a) **HRP proliferation** (DNA synthesis) and **b**) **apoptosis** (DNA fragmentation), following 8-day exposure to CM obtained by culturing Bv-2 cells in physiological conditions (basal-CM), SST (SST-CM), LPS (LPS-CM) and LPS + SST (LPS+SST-CM), as compared with HRP cultured in physiological conditions without CM (noCM). Mean of 5 experiments, * = p<0.05 vs noCM, \$ = p<0.05 vs LPS-CM



Fig. 3. Expression of **pro-inflammatory markers** by HRP following 8-day exposure to CM obtained by culturing Bv-2 cells in physiological conditions (basal-CM), SST (SST-CM), LPS (LPS-CM) and LPS + SST (LPS+SST-CM), as compared with HRP cultured in physiological conditions without CM (noCM). **a**) iNOS, **b**) TNF α , densitometric analysis normalized to α -tubulin, mean of 5 experiments ± SD, *p <0.05 vs noCM and basal-CM, # p<0.05 vs SST-CM, \$ p<0.05 vs LPS-CM; **c**) representative image of one of the Western blots.



Fig. 4. Expression of **pro-apoptotic factors** in HRP following 8-day exposure to CM obtained by culturing Bv-2 cells in physiological conditions (basal-CM), SST (SST-CM), LPS (LPS-CM) and LPS + SST (LPS+SST-CM), as compared with HRP cultured in physiological conditions without CM (noCM). **a**) FasL, **b**) ratio active to pro-caspase-8, **c**) ratio tBid to Bid, **d**) Bax, **e**) p53. Densitometric analysis normalized to α -tubulin, mean of 5 experiments ± SD, *p <0.05 vs noCM and basal-CM, # p<0.05 vs SST-CM, \$ p<0.05 vs LPS-CM. **f**) Representative image of one of the Western blots.



Fig. 5. Expression of **pro-survival factors** in HRP following 8-day exposure to CM obtained by culturing Bv-2 cells in physiological conditions (basal-CM), SST (SST-CM), LPS (LPS-CM) and LPS + SST (LPS+SST-CM), as compared with HRP cultured in physiological conditions without CM (noCM). **a**) BclxL, **b**) ratio pAkt to Akt. Densitometric analysis normalized to α -tubulin, mean of 5 experiments ± SD, *p <0.05 vs noCM, £ p<0.05 vs basal-CM, # p<0.05 vs SST-CM, \$ p<0.05 vs LPS-CM. **c**) Representative image of one of the Western blots.



Fig. 6. Translocation of active caspase-8 from HRP cytoplasm into the nucleus, following 8-day exposure to CM obtained by culturing Bv-2 cells in physiological conditions (basal-CM), SST (SST-CM), LPS (LPS-CM) and LPS + SST (LPS+SST-CM), as compared with HRP cultured in physiological conditions without CM (noCM). **a**, **b**) Expression of active caspase-8 into cytoplasmic and nuclear fractions, densitometric analysis expressed as ratio active to procaspase-8, mean of 5 experiments ± SD, *p <0.05 vs noCM, £ p<0.05 vs basal-CM, # p<0.05 vs SST-CM, \$ p<0.05 vs LPS-CM. **c**, **d**) Representative images of one of the Western blots. β-actin and histone H3 expression were used as controls of cytoplasmic and nuclear fractions, respectively. **e**, **f**) Immunofluorescence staining for active caspase-3 (**e**) and active caspase-8 (**f**) (*green*). Nuclei are counterstained with DAPI (*blue*). Caspase-3 remains in the cytoplasm in all cases, while translocation of active caspase-8 into the nucleus is evident in LPS-CM, with SST+LPS-CM partially counteracting this effect. **g**) Dapi, FITC and merge images of the most relevant cases, as regards active caspase-8 IF staining, to underline the nuclear translocation. Magnification 400x.