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Beltramo, Elena; Lopatina, Tatiana; Mazzeo, Aurora; Arroba, Ana I; Valverde, Angela M; Hernández, Cristina; Simó, Rafael; Porta, Massimo. Effects of the neuroprotective drugs somatostatin and brimonidine on retinal cell models of diabetic retinopathy. ACTA DIABETOLOGICA. None pp: 1-8.
DOI: 10.1007/s00592-016-0895-4

Effects of the neuroprotective drugs somatostatin and brimonidine on retinal cell models of diabetic retinopathy

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Abstract

Aims: Diabetic retinopathy (DR) is considered a microvascular disease but recent evidence has underlined early involvement of the neuroretina with interactions between microvascular and neural alterations. Topical administration of somatostatin (SST), a neuroprotective molecule with antiangiogenic properties, prevents diabetes-induced retinal neurodegeneration in animals. The α_2-adrenergic receptor agonist brimonidine (BRM) decreases vitreoretinal vascular endothelial growth factor and inhibits blood-retinal barrier breakdown in diabetic rats. However, SST and BRM effects on microvascular cells have not yet been studied. We investigated the behaviour of these drugs on the crosstalk between microvasculature and neuroretina.

Methods: Expression of SST receptors 1-5 in human retinal pericytes (HRP) was checked. We subsequently evaluated the effects of diabetic-like conditions (high glucose and/or hypoxia) with/without SST/BRM on HRP survival. Endothelial cells (EC) and photoreceptors were maintained in the above conditions and their conditioned media (CM) used to culture HRP. Vice versa, HRP-CM was used on EC and photoreceptors. Survival parameters were assessed.

Results: HRP express the SST receptor 1 (SSTR1). Glucose fluctuations mimicking those occurring in diabetic subjects are more damaging for pericytes and photoreceptors than stable high glucose and hypoxic conditions. SST/BRM added to HRP in diabetic-like conditions decrease EC apoptosis. However, neither SST nor BRM changed the response of pericytes and neuroretina-vascular crosstalk under diabetic-like conditions.

Conclusions: Retinal pericytes express SSTR1, indicating that they can be a target for SST. Exposure to SST/BRM had no adverse effects, direct or mediated by the neuroretina, suggesting that these molecules could be safely evaluated for the treatment of ocular diseases.

Keywords: diabetic retinopathy, somatostatin, brimonidine, pericyte, endothelial cell, photoreceptor cell.

Abbreviations

BRB, blood-retinal barrier
BRM, brimonidine
DME, diabetic macula edema
DR, diabetic retinopathy
EC, endothelial cells
HG, high glucose concentrations
HMEC, human microvascular endothelial cells
Hypo, hypoxic conditions
intHG, intermittent high glucose concentrations
NG, physiological glucose concentrations
NMDA, N-methyl-D-aspartate
PDR, proliferative diabetic retinopathy
RT-PCR, real-time PCR
SST, somatostatin
SSTR, somatostatin receptor(s)
VEGF, vascular endothelial growth factor

Introduction

Diabetic retinopathy (DR), a sight-threatening complication of diabetes, has been long described as a microvascular disease. Loss of retinal capillary pericytes and thickening of the basement membrane, well-known key-events in its pathogenesis, may lead to failure of control on endothelial proliferation and, consequently, abnormal angiogenesis [1,2]. In the last years, however, strong evidence has pointed out to the involvement of the neuronal part of the retina in the early stages of the disease [3-5]. Glial activation and neuronal apoptosis, hallmarks of retinal neurodegeneration, have been shown in the retina of diabetic human donors even before any clinical observation of microaneurysm development [6]. Elevated levels of glutamate, the main excitatory neurotransmitter in the retina, may result in overstimulation and be implicated in the so-called “excitotoxicity” leading to neurodegeneration [7], while oxidative stress [8,9], increase of advanced glycation end product formation [9,10], and activation of the renin-angiotensin system [11,12] are shared features of the diabetes-induced microvascular and neural alterations.

Pericytes play a central role in the pathogenesis of DR, since, though part of the microvasculature and strictly involved in the pathogenesis of several vessel abnormalities [2, 13], in the capillaries they behave like a sort of bridge between endothelium and the neuronal part of the retina. Pericytes modulate vascular permeability, including the blood-brain and blood-retinal barriers (BRB) and regulate endothelial cell (EC) proliferation, migration and survival [2]. On the one hand, EC are exposed to the complex signals deriving from the blood stream and can therefore influence pericytes; on the other, pericytes receive, and transmit to the endothelium, signals from the basement membrane and the surrounding tissues, in particular the neuroretina.

Somatostatin (SST) is one of the most important neuroprotective molecules synthesized by the retina, acting in an autocrine way through several pathways, such as intracellular $Ca2^{+}$ signalling, nitric oxide function, and glutamate release from the photoreceptors [14]. Moreover, SST has antiangiogenic properties [15], regulates various ion/water transport systems [16], and thus could also prevent proliferative DR (PDR) and diabetic macular edema (DME). In the early stages of DR, downregulation of SST has been described [6], while the intravitreal injection of SST and SST analogues protects the retina from neurotoxicity [17]. Topical administration of SST in eye drops was shown to prevent diabetes-induced retinal neurodegeneration, thus overcoming concern about the use of eye-drops for posterior chamber diseases and opening a new route for non-invasive DR prevention [5].

The selective α_2-adrenergic receptor agonist brimonidine (BRM) may also have protective effects in retinal damage, since it has been shown to decrease intracellular $Ca2^{+}$ due to glutamate excitotoxicity in cultured ganglion cells [18] and to preserve retinal function in rat models of transient retinal ischemia [19]. Moreover, BRM decreased vitreoretinal vascular endothelial growth factor (VEGF) and inhibited BRB breakdown in diabetic rats, suggesting a role for the treatment of ocular diseases associated with BRB leakage, such as DR and DME [20].

Despite the strict interactions between the microvascular and neuronal parts of the retina, SST and BRM effects on vascular cells remain to be understood. Therefore, our aim was to investigate the potential effects

of SST and BRM on EC and pericytes cultured in diabetic-like conditions (high glucose and/or hypoxia) and in co-culture cell models mimicking the crosstalk between the microvascular and neural sides of the retina.

Methods

Cell models

Home-immortalized human retinal pericytes (HRP), commercially purchased immortalized human microvascular endothelial cells (HMEC, Lonza) and the immortalized mouse photoreceptor cell line 661W (kindly provided by Muayyad Al-Ubaidi, University of Oklahoma, Norman, OK, USA) were used.

HRP and 661W cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma Aldrich), HMEC in EBM-2 Medium (Lonza). All media had a 5.6 mmol/L D-glucose concentration (physiological condition, NG). High glucose concentrations (HG) were obtained by adding D-glucose to a final concentration of 28 mmol/L. Cells were also grown in intermittent HG conditions (48hr HG/ 48hr NG twice, intHG), because we have previously demonstrated that human pericytes are affected by intermittent HG conditions, rather than stable HG [21]. All conditions were maintained for 8 days. Hypoxic conditions (hypo) were obtained by keeping cultures in a 5%CO_2 / 94%N_2 /1%O_2 gas mixture for the last 48 hrs. Following dose-response experiments, SST (a generous gift by BCN Peptides) concentration was set at 10^{-7} M and BRM (Sigma Aldrich) at 10^{-8} M. To evaluate the respective influence of one cell type on the other(s), conditioned media (CM) from the final two days were collected and used at 50% concentration to culture the other(s) cell type(s).

Survival parameters

To evaluate survival parameters, cells were counted in Bürker chambers after Trypan blue staining by 2 independent operators, proliferation was measured as BrdU incorporation (Cell Proliferation ELISA BrdU kit, Roche) and apoptosis as DNA fragmentation (Cell Death Detection ELISA^{PLUS} kit, Roche). Results were checked through a fluorescent/chemioluminescent assay which measures viability, cytotoxicity and apoptosis (caspase 3/7 activity) in the same well (ApoTox-Glo™ Triplex Assay, Promega).

Expression of SST receptors

The presence of SST receptors in HRP was evaluated by Real Time quantitative PCR (RT-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). Primers for hSSTR1 were: forward 5'-CGCTGGCTGGTGGGCTTCGTGTTG-3', reverse 5'-CGCCGCCGGACTCCAGGTTCTCAG-3'; hSSTR2: forward 5'-CATGGACATGGCGGATGAG-3', reverse 5'-CTCAGATACTGGTTTGGAG-3'; hSSTR3: forward 5'-GCGAGCCGGCTTCATCATCTACAC-3', reverse 5'-GACCCGGCCGTTCATCTCCTTC-3'; hSSTR4: forward 5'- TGGTCGGCAGTCTTCGTGGTCTAC-3', reverse 5'- CTTGCGGCCGGGTTCTGGT-3'; hSSTR5: forward 5'- GCGGCCTGGGTCCTGTCTCT-3', reverse 5'-CCCCCGCCTGCACTCTCAC-3'. RNA expression was normalized against the small nuclear RNA RNU6B.

Results of SSTR expression were checked by Western blot analysis. Cells were lysed using M-PER Mammalian Protein extraction reagent (Pierce) added with 10 µl/ml protease inhibitor cocktail kit (Pierce). Protein concentration was measured using Bradford method. 30µg proteins were loaded on pre-cast gels (4–15% Mini-PROTEAN® TGX™ Precast Gel, Biorad), separated by electrophoresis and transferred to nitrocellulose membranes. Immunoblotting was performed by incubating the membranes with 1 µg/ml Anti-Somatostatin Receptor 1 antibody (ab2366, Abcam). Secondary antibody was goat polyclonal to Rabbit IgG - H&L - Pre-Adsorbed horseradish peroidase (Abcam) at a dilution of 1/3000, developed using the ECL technique. The relative signal strength was quantified by densitometric analysis (1D Image Analysis System, Kodak), and values normalized against β-actin.

Immunofluorescence was performed after fixation of cells with ice-cold methanol, by overnight incubation at 4°C with the above-mentioned anti-SSTR1 antibody (1 µg/ml). Secondary antibody was FITC-conjugated goat anti-rabbit IgG (Sigma Aldrich) used at a 1/1000 dilution for 1h. DAPI was used to blue-stain the cell nuclei.

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 ± SD of 5 independent experiments, normalized against control (NG).

Results

SST receptors, especially SSTR1 and 2, are widely expressed in the retina [22, 23]. EC are known to express mostly SSTR2 and 5 [24], but nothing is known about SSTRs in pericytes. Our first step was therefore to check if and which SST receptor(s) were expressed by HRP. Real-time PCR showed that HRP strongly express SSTR1 and, to a much lower degree, SSTR4 and 5 (**Fig. 1a**). Western blot (**Fig. 1b**) and immunofluorescence (**Fig. 1c**) analysis confirmed SSTR1 expression in HRP.

We evaluated the effects of SST and BRM on pericytes, since they are the earliest vascular cells affected by DR and they constitute a sort of bridge between the neuroretina and the retinal microvessels. In agreement with previous results of our group, proliferation decreased (-19.3%, $p<0.05$ vs NG) and apoptosis increased (+12.1%, $p<0.05$ vs NG) in HRP exposed to intHG [20,25]. Hypoxic conditions decreased HRP proliferation independently of glucose concentrations in the media (-26.2%, $p<0.05$ vs NG) (**Fig. 2a**) and had a synergic effect with intHG in decreasing proliferation (-32.8%, $p<0.05$ vs NG) and increasing apoptosis (+50.8%, $p<0.05$ vs NG) (**Fig.2b**). SST and BRM did not exert significant effects on retinal pericytes (**Fig. 2 a-b**).

EC-mediated effects of BRM and SST on pericytes were checked. HMEC were cultured in NG, HG, intHG with/without SST/BRM for 8 days, media from the last 2 days collected, and pericytes exposed to them for 8 days. We found that HRP grow better (+15% average in all cases, $p<0.05$ vs ctrl) and undergo less

apoptosis (-11.3% in NG-CM, -17.75% in HG-CM, -23.4% in intHG-CM, $p<0.05$ vs ctrl, in all cases) when cultured in EC-produced CM. SST and BRM, when added to EC while producing CM did not show any relevant effect on HRP proliferation or apoptosis (**Fig. 3 a-b**).

Similarly to pericytes, HMEC also grow better when exposed to physiological conditioned media from HRP than in standard medium without CM (+15.8%, $p<0.05$). This increase in proliferation is augmented when EC are cultured in CM from HRP cultured in stress conditions (HG/intHG) (+43.5 and +66.9 respectively, $p<0.05$ vs ctrl), indicating that HRP grown in a diabetic-like milieu may lose their proliferative control on EC proliferation. In this set of experiments, SST added to HG seems to enhance the proliferative effect of HRP-CM, while BRM in physiological conditions may increase HRP control on EC proliferation (**Fig. 3c**). Not surprisingly, HMEC cultured in HRP-CM underwent apoptosis more than in standard medium (+44.5%, $p<0.05$), while the previous stimulation of HRP with HG added with SST/BRM decreased EC apoptosis (-45.8 and – 25.5 respectively, $p<0.05$ vs ctrl) (**Fig. 3d**). Hypoxic conditions did not change significantly these parameters (data not shown).

Subsequently, we verified the effects of the addition of SST and BRM on the microvascular-neuroretina crosstalk, by culturing HRP or 661W cells in diabetic-like conditions and using their respective conditioned media to culture the other cell type. HRP proliferation decreased (-19.8%, $p<0.05$ vs NG-CM) after exposure to intHG-CM from 661W cells, while apoptosis remained substantially stable (**Fig. 4 a-b**).CM-mediated hypoxic conditions did not have any effect (data not shown).
661W cells proliferation decreased (-30.0%, $p<0.005$ vs ctrl) and apoptosis increased (+31.8%, $p<0.05$ vs ctrl) in intHG conditions. 661W cells proliferated less when exposed to CM from HRP (-40.4% in NG-CM vs ctrl, $p<0.05$). When cultured in intHG-CM from HRP, 661W cells showed increased apoptosis (+57.4%, $p<0.005$ vs NG-CM) (**Fig. 4 c-d**). CM from HRP in hypoxic conditions did not increase intHG-CM effects on 661W cells (data not shown). SST and BRM exerted no effect, either positive or negative, in all cases.

Discussion

In this work, we show, for the first time in our knowledge, the expression of SSTR1 by human retinal pericytes, indicating that these cells can represent a target for somatostatin. In addition, we demonstrate that glucose fluctuations, mimicking what happens in the diabetic subject, are more damaging for pericytes than hypoxia, but these two stress conditions may act synergistically. Finally, we provide evidence that the neuroprotective drugs SST and BRM do not exert any effect, direct or mediated by the neuroretina, in these experimental settings. These results suggest that the potential beneficial effects of SST and BRM in retinal microangiopathy are not mediated by the crosstalk between the neuroretina and the microvessels.

SST and BRM, administered as eye-drops, could be an efficient and non-invasive new therapeutical approach to treat diabetic-induced neurodegeneration [5]. In fact, there is a need for early non-invasive pharmacological prevention and/or treatment of DR, since the existing methods are quite aggressive (intravitreal injections, laser photocoagulation). Nevertheless, besides the positive neuroprotective effects of these drugs on neuroretina in experimental models [5,26], it was also necessary to examine all potential

beneficial activity and, meanwhile, rule out any possible adverse effect on the retinal microvessels, which are closely linked to the neuroretina and strongly affected by DR.

The presence in the retina of somatostatin receptors, especially SSTR1 and 2, is well known, with differences among the different cell types [22,23]. EC express mostly SSTR2 and 5 [24], but nothing was known until now about SSTRs in pericytes. Our finding that pericytes in basal conditions express mostly SSTR1 is rather surprising, since SSTR1 acts as an autoreceptor in the neuroretina by modulating SST levels [27] and has an inhibitory effect on SSTR2 [28], which, in turn, is known to have anti-angiogenic properties against hypoxia-induced retinal degeneration [29]. As pericytes exert a control on EC proliferation and, consequently, on abnormal angiogenesis in PDR [1,2], one would expect them to rather express SSTR2.

Loss of pericytes as a consequence of hyperglycaemia is well-described in the literature [30], and similar effects of hypoxia have been observed more recently [31]. In this work, we demonstrate that hypoxia plays a synergistic role with hyperglycaemia in reducing pericyte proliferation and increasing their apoptosis. Depletion of EC-controlling pericytes from the vessel wall, together with hypoxia-induced VEGF upregulation [32] and the direct deleterious contribution of high ambient glucose, all together may lead to vessel sprouting and abnormal angiogenesis.

Despite the presence of the SSTR1, we found that direct addition of somatostatin does not exert any effect on pericyte proliferation or apoptosis, in basal as well as in diabetic-like conditions. This may be seen as a positive finding in the microvascular milieu, since early loss of pericytes is one of the hallmarks of DR and it is known that addition of SST to active-proliferating cells causes cell growth arrest [33-35], and even, in some cell types, apoptosis and cell death, through activation or upregulation of the pro-apoptotic proteins p53 and Bax [36].

Regarding the crosstalk among the different components of the retina, we observed, not surprisingly, that both endothelial cells and pericytes grow better when they are exposed to media obtained by the other cell type than when they are cultured in fresh medium. This is consistent with our previous observations [37] and is explained by the complex exchange of factors between the two cell types, that in physiological conditions live in strict contact and in constant equilibrium. For instance, PDGF released by the endothelium is necessary to pericyte growth and vessel stabilization, while VEGF released and controlled by the pericytes plays a key-role in EC proliferation and angiogenesis [30].

We found that intHG conditions, both direct and mediated by pericytes, decrease photoreceptor proliferation and increase their apoptosis, a behaviour similar to that shown by pericytes. As pericytes are sensitive to intermittent, but not stable, high glucose [21,25], it can be hypothesized that pericytes in stress conditions may release soluble factor(s) affecting not only endothelium, but also neighbouring neural cells. As a matter of fact, pericytes themselves, living in the outer part of the retinal capillaries, are subjected to the influence of paracrine signalling from the surrounding tissue. We have recently demonstrated that extracellular vesicles released by the mesenchymal stem cells in diabetic-like conditions are able to enter the pericytes, causing their detachment from substrate and migration [38], and to stimulate angiogenesis *in vitro*, down-regulating

the expression of miR-126, and thus leading to increased release of angiogenic molecules, such as VEGF and HIF-1α [39].

We also observed increased proliferation and decreased apoptosis of endothelial cells cultured with conditioned media obtained by culturing pericytes in hyperglycaemic conditions with the addition of SST. This could be ascribed to an arrest of cell growth in pericytes, due to somatostatin [34], acting in a synergistic way with intermittent HG. Since pericytes and EC live in a delicate equilibrium, with the former exerting *in vivo* an active control on endothelium survival and proliferation [2], inhibition of pericyte growth can have as a consequence a loss of this control, leading to abnormal angiogenesis. However, since DR is characterized also by acellular capillaries, the increased proliferation and decreased apoptosis observed in EC exposed to CM from pericytes in intHG+SST, could be also seen as a positive attempt to by-pass this event.

Pericytes express the α_2-adrenergic receptors [40,41], therefore they could potentially be a target of BRM, an α_2-adrenergic agonist, which was shown to reduce intravitreal VEGF and inhibit BRB breakdown in animal models of DR [20]. However, we could not find any beneficial effect of the addition of BRM to pericytes grown in hyperglycaemic conditions. The mechanism of action of BRM in counteracting VEGF overexpression and BRB leakage has been attributed to a modulation of the function of N-methyl-D-aspartate (NMDA)-type glutamate receptors, which are present in neural retinal cells [20]. These receptors have only been detected in pericytes after they were induced *in vitro* to differentiate into neural-like cells [42]. Therefore, it is reasonable to hypothesize that the lack of effect of BRM on pericytes is due to the absence of NMDA receptors. This could also explain why we did not find any effect of BRM on pericyte/endothelial cell and pericyte/neural cell crosstalk models.

A limit of our study is the use of a mouse photoreceptor model, along with human-derived microvascular cells, due to the non-availability of human neuronal cell models. Moreover, we are available that photoreceptors represent only one type of neuroretinal cells. However, recent findings report that DR provokes damages, of different grade or intensity, in all retinal layers [43, 44], with photoreceptors highly involved and associated with deep retinal capillary nonperfusion [45].

In conclusion, since neurodegeneration and vascular abnormalities are linked in the pathogenesis of early DR, we have examined the behaviour of the neuroprotective drugs SST and BRM on the retinal microvasculature and on its exchanges with the surrounding neural tissue. We have demonstrated expression of the SSTR1 in human retinal pericytes, indicating that they can be a target for this molecule. Nevertheless, exposure to SST or BRM had no effects, neither direct nor mediated by the neuroretina. Our results suggest that these molecules could be safely evaluated in further steps aimed at understanding their potential beneficial action for the treatment of ocular diseases.

Acknowledgements

This research was supported by the EUROCONDOR project, grant agreement number 278040, funded by the European Commission's Seventh Framework Programme (theme FP7-HEALTH-2011.2.4.3-1). This

publication reflects the views only of the Authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

Conflict of interest

Elena Beltramo, Tatiana Lopatina, Aurora Mazzeo, Ana I Arroba, Angela M Valverde, Cristina Hernández, Rafael Simó, and Massimo Porta declare that they have no conflicts of interest.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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Figures

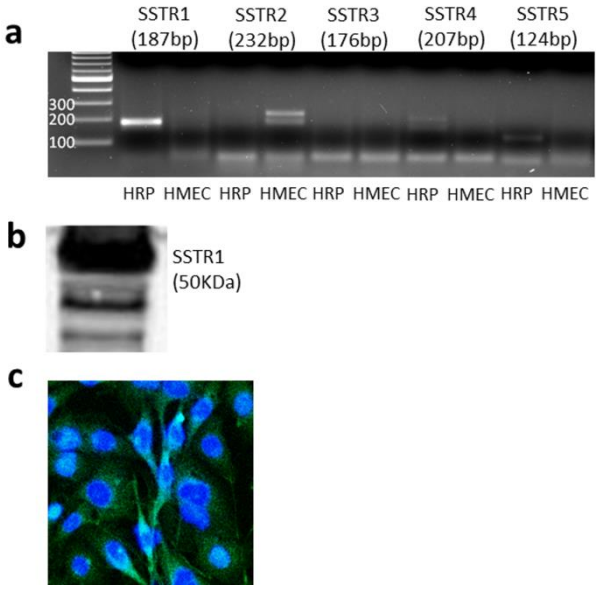

Fig. 1 HRP express SSTR1, while HMEC SSTR2: **a**) RT-PCR for the five SST receptors in HRP and HMEC, used as a control; **b**) Western blot and **c**) immunofluorescence staining for SSTR1 in HRP

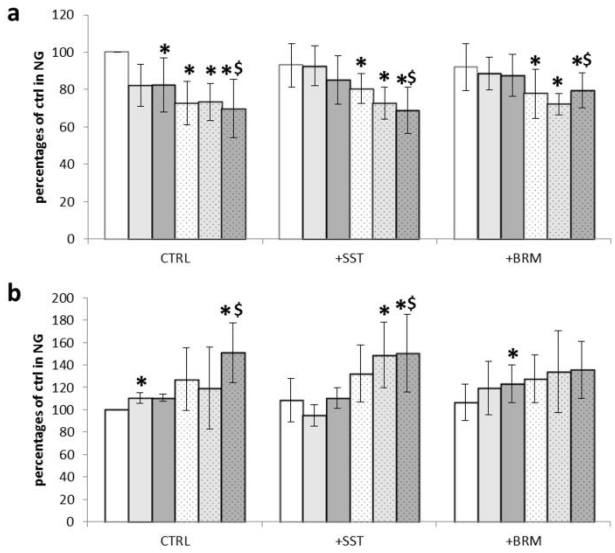

Fig. 2 a) **HRP proliferation** and **b**) **HRP apoptosis**, in NG, HG, intHG with/without hypoxia, and following addition of SST or BRM. *White bars*: NG; *light grey bars*: HG; *dark grey bars*: intHG; *dotted white bars*: NG+hypo; *dotted light grey bars*: HG+hypo; *dotted dark grey bars*: intHG+hypo. N=5, *=p<0.05 vs ctrl (NG), $ = p<0.05 vs intHG

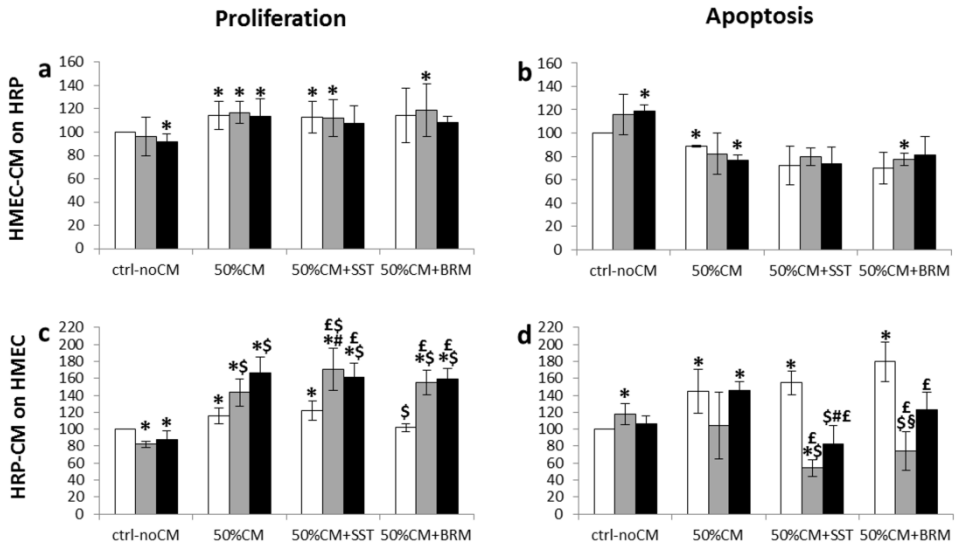

Fig. 3 a) HRP proliferation after exposure to HMEC-CM; **b**) HRP apoptosis after exposure to HMEC-CM; **c**) HMEC proliferation after exposure to HRP-CM; **d**) HMEC apoptosis after exposure to HRP-CM. *White bars*: NG; *grey bars*: HG; *black bars*: intHG. CM were obtained by culturing cells in the relevant media, collecting media of the last 2 days and exposing the other cell type to 50% CM and 50% normal medium with NG, HG or intHG, respectively. N=5, data expressed as percentages of ctrl (NG-noCM). * p<0.05 vs ctrl-noCM, $ p<0.05 vs NG-CM, £ p<0.005 vs NG+SST/BRM-CM, # p<0.05 vs HG-CM, § p<0.05 vs intHG-CM

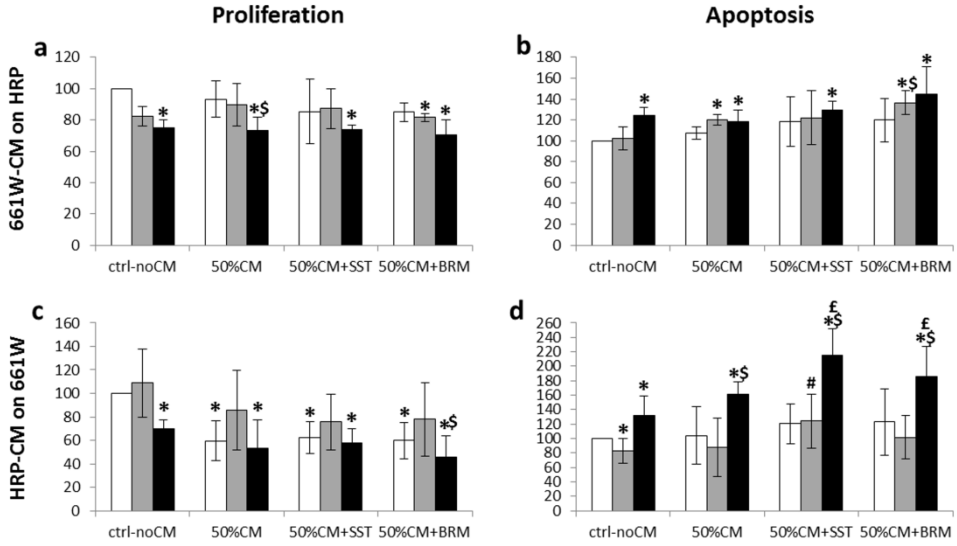

Fig. 4 a) HRP proliferation after exposure to 661W-CM; **b**) HRP apoptosis after exposure to 661W-CM; **c**) 661W proliferation after exposure to HRP-CM; **d**) 661W apoptosis after exposure to HRP-CM. *White bars*: NG; *grey bars*: HG; *black bars*: intHG. CM were obtained by culturing cells in the relevant media, collecting media of the last 2 days and exposing the other cell type to 50% CM and 50% normal medium with NG, HG or intHG, respectively. N=5, data expressed as percentages of ctrl (NG-noCM). * p<0.05 vs ctrl-noCM, $ p<0.05 vs NG-CM, £ p<0.005 vs NG+SST/BRM-CM, # p<0.05 vs HG-CM, § p<0.05 vs intHG-CM