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Abstract

Aims: Although diabetic retinopathy has long been considered a microvascular complication, retinal neurodegeneration and inflammation may precede its clinical manifestations. Despite all research efforts, the primary treatment options remain laser photocoagulation and anti-vascular endothelial growth factor (VEGF) intravitreal injections, both aggressive and targeting the late stages of the disease. Medical treatments addressing the early phases of diabetic retinopathy are therefore needed. We aimed at verifying if thiamine and fenofibrate protect the cells of the inner blood-retinal barrier from the metabolic stress induced by diabetic-like conditions.

Methods: Human microvascular endothelial cells (HMECs), retinal pericytes (HRPs) and Müller cells (MIO-M1) were cultured in intermittent high glucose (intHG) and/or hypoxia, with addition of fenofibrate or thiamine. Modulation of adhesion molecules and angiogenic factors was addressed.

Results: Integrins β1/αVβ3 and ICAM1 were upregulated in HMECs/HRPs cultured in diabetic-like conditions, as well as metalloproteases MMP2/9 in HRP, with a reduction of their inhibitor TIMP1; MMP2 increased also in HMEC, and TIMP1 decreased in MIO-M1. VEGF and HIF-1α were strongly increased in HMEC in intHG+hypoxia, and VEGF also in HRP. Ang-1/2 augmented in HMEC/MIO-M1, and MCP-1 in HRP/MIO-M1 in intHG+hypoxia. Thiamine was able to normalize all such abnormal modulations, while fenofibrate had effects in few cases only.

Conclusions: We suggest that endothelial cells and pericytes are more affected than Müller cells by diabetic-like conditions. Fenofibrate shows a controversial behavior, potentially positive on Müller cells and pericytes, but possibly detrimental to endothelium, while thiamine confirms once more to be an effective agent in reducing diabetes-induced retinal damage.

Keywords: diabetic retinopathy, inner blood-retinal barrier, thiamine, fenofibrate, hypoxia, hyperglycemia

Introduction

Diabetic retinopathy (DR) has long been considered a microvascular complication. However, retinal neurodegeneration and inflammation have recently come into focus as events preceding the clinical manifestations of this complication [1]. Despite the efforts of the last decades, the primary options for DR treatment remain laser photocoagulation and anti-vascular endothelial growth factor (VEGF) intravitreal injections, both invasive and targeting the late stages of the disease. Therefore, medical treatments addressing the early phases of DR are strongly needed.

Among all actors involved in the pathogenesis of early DR, cells belonging to the inner blood-retinal barrier (iBRB) are primarily involved. While endothelial cells (ECs) are the first target for potentially damaging circulating metabolites, pericytes play a central role in iBRB maintenance [2]. Müller cells, on the other hand, constitute the link between the vascular and neural sides of the retina. In DR, they contribute to neuronal dysfunction, chronic inflammation and production of pro-angiogenic factors leading to neovascularization [3].

Thiamine (T), an essential cofactor for key-enzymes involved into intracellular glucose metabolism, acts as an anti-oxidant against hyperglycemic-induced damage. Diabetic patients are subjected to renal loss of thiamine via proximal tubules, with reduction in thiamine availability and transketolase activity [4]. Thiamine supplementation reduces progression of DR and nephropathy in diabetic animals [5, 6]. A major role for thiamine transporter-2 (THTR2) in thiamine transport inside retinal cells and its involvement in high glucose-induced damage and impaired thiamine availability has been recently postulated [7].

Fenofibric acid (FA), the active form of fenofibrate, is commonly used to treat dyslipidemia. Its possible role in reducing the progression of DR was postulated by two major clinical trials, the FIELD study [8] and the ACCORD-Eye study [9], but the mechanisms of action are still poorly understood [10]. FA seems to act as an anti-inflammatory agent [11, 12], but also as an anti-angiogenic element, by inhibiting neovascularization [13].

The aim of this study was to verify if thiamine and fenofibrate protect the iBRB from the metabolic stress induced by diabetic-like conditions, addressing their potential involvement in the modulation of adhesion molecules and angiogenic factors.

Methods

Cell cultures

A human retinal pericyte (HRP) line was stabilized in our laboratory [14]. Human microvascular endothelial cells (HMECs) were purchased from ATCC (Cat#CRL-3243, RRID:CVCL_0307), and the human Müller cell line Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1) (RRID:CVCL_0433) obtained from the UCLB licensing portal XIP (London, UK) [15]. HRP and MIO-M1 cells were cultured in DMEM+10%FCS; HMEC in EBM-2 growth medium (Lonza) for expansion and DMEM+10%FCS for experiments.

Cells were cultured for 8 days in physiological conditions (NG, 5.6 mmol/l D-glucose), or intHG (48hr high glucose, 28 mmol/l /48hr NG, twice), to better mimic the diabetic microenvironment, since we had previously demonstrated that human pericytes are more affected by intermittent high glucose conditions [16]. 50 µmol/l thiamine (T) and 100 µmol/l fenofibric acid (FA) (concentrations chosen on the basis of previous reports [7, 10]) were added during the whole 8-day incubation. 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.

Reagents for cell cultures were purchased from Sigma-Aldrich, unless otherwise stated.

FACS analysis

Expression of integrins β1 (CD29), αvβ3 (CD51/61) and Intercellular Adhesion Molecule-1 (ICAM-1/CD54) was measured through a CytoFLEX flow cytometer (Beckman Coulter), with CytExpert software. Antibodies (dilution 1:20) were: anti-CD51/CD61-FITC (ThermoFisher Scientific Cat#11-0519-42, RRID:AB_1907402), anti-CD29-FITC (ThermoFisher Cat#11-0299-41, RRID:AB_2043830), anti-ICAM-1-PE (Thermo Fisher Cat#12-0549-42, RRID:AB_10598517).

Western blot analysis

Expression of metalloproteases 2/9 (MMP2/MMP9), their tissue inhibitor TIMP1, VEGF and hypoxia-inducible factor 1α (HIF-1α) was evaluated by Western blot. To extract total proteins, cells were lysed using *M-PER Mammalian Protein extraction reagent* (ThermoFisher) with 10 µl/ml *protease inhibitor cocktail kit* (ThermoFisher), extracts cleared by centrifugation at 20,000 g for 15 min at 4°C and protein content measured through Bradford reaction. 30 µg protein were loaded on pre-cast gels (4–20% Mini-PROTEAN® TGX™ Precast Gel, Bio-Rad), separated by electrophoresis and transferred to nitrocellulose membranes. Immunoblotting was performed using specific antibodies (dilution 1:500): anti-MMP2 (ThermoFisher, Cat#MA5-17122, RRID:AB_2538593), anti-MMP9 (ThermoFisher, Cat#MA5-15888, RRID:AB_11157869), anti-TIMP1 (ThermoFisher, Cat#MA5-23756, RRID:AB_2609828), anti-VEGF (SantaCruz Biotechnology, Cat#sc-7269, RRID:AB_628430), anti-HIF-1α (SantaCruz, Cat#sc-13515, RRID:AB_627723). Bands were visualized using the enhanced chemiluminescence (ECL) WB protocol (Merck-Millipore), and the relative signal strength quantified by densitometric analysis (1D Image Analysis System, Kodak) and normalized against β-actin (Sigma-Aldrich, Cat#A5316, RRID:AB_476743).

ELISA

Release of Angiopoietin 1 and 2 (Ang-1/Ang-2), platelet-derived growth factor B (PDGF-B) and Monocyte Chemoattractant Protein 1 (MCP-1) was measured by ELISA. Kits used were: *Angiopoietin 1 Human ELISA Kit* (ThermoFisher, Cat# EHANGPT1), *Angiopoietin-2 Human ELISA Kit* (ThermoFisher, Cat#KHC1641), *PDGF-BB Human ELISA Kit* (ThermoFisher, Cat#EHCSRP2), *Human MCP-1 Platinum ELISA Kit* (Invitrogen, Cat#BMS281CE).

Vessel-like formation assay

15,000 HRPs and 15,000 HMECs were seeded together onto Matrigel-coated 24-well plates and cultured in DMEM. 30,000 HRPs or 30,000 HMECs seeded alone served as controls. After 24 h, phase-contrast photos at 100x of five random fields/well were captured. The total length of the structures in each field was measured using the MicroImage analysis system (Casti Imaging, Venice, Italy), and the mean of 5 fields/well calculated. Each measure was performed in duplicate wells and expressed as ratio of control.

Statistical analysis

Statistical comparison was performed by ANOVA with Bonferroni *post-hoc* correction. Results were expressed as mean ± SD of 5 independent experiments, as previously established through a power analysis, and normalized against cells cultured in physiological conditions (NG). The SPSS software version 24.0 (IBM) was used for statistical analysis.

Results

Adhesion molecules

Since pericyte detachment/migration is a key-event in DR pathogenesis, we examined the expression of adhesion molecules, affected by diabetes: integrin β1, integrin αVβ3 and ICAM-1. All of them (**Fig. 1**) showed an increase in HMEC cultured in intHG+hypo ($p<0.05$ vs NG ctrl), with integrin β1 also increased in NG+hypo ($p<0.05$ vs NG ctrl) (**Fig. 1 a, d, g**). Thiamine normalized these altered expressions, while FA had

positive effects on ICAM-1 only (**Fig. 1g**). In HRP, integrin β1 expression was augmented in intHG and intHG+hypo ($p<0.05$ vs NG ctrl) (**Fig. 1b**), and ICAM-1 in intHG+hypo ($p<0.05$ vs NG ctrl) (**Fig. 1h**); all of them were normalized by thiamine supplementation, but not FA. Expression of integrins β1, αVβ3 and ICAM-1 was unchanged in Müller cells, independently of treatment **(Fig. 1c,f,i)**.

Matrix metalloproteases

We evaluated the expression of MMP2 and MMP9, and their inhibitor TIMP1, because of their strict involvement in the mechanisms of the iBRB disruption [17, 18] (**Fig. 2**). Both MMP2 and MMP9 were increased, and TIMP1 reduced, in HRP cultured in intHG/intHG+ hypo ($p<0.05$ vs NG ctrl). IntHG and hypoxia worked synergistically in increasing MMP2 ($p<0.05$ for intHG+hypo vs intHG) (**Fig. 2b,e,h**). Thiamine was able to correct altered MMP2/9 expression and, partly, TIMP1 (**Fig. 2b,e**), while FA normalized MMP9 expression, when added to intHG+hypo (**Fig. 2e**). MMP2 expression increased also in HMEC cultured in intHG+hypo and was corrected by T, but not FA (**Fig.2a**). As regards Müller cells, no differences were found in MMP2/MMP9 expression in diabetic-like conditions, but T addition decreased their expression in comparison with basal conditions (**Fig. 2c,f**), while TIMP1 expression decreased in hypoxic treatment (**Fig. 2i**).

Angiogenic/inflammatory factors

DR is characterized by vessel sprouting, abnormal angiogenesis and inflammation. We studied a panel of factors, involved in these processes (**Fig. 3-4**). The most affected were VEGF and HIF-1α (**Fig. 3**), dramatically increased (2.5/3-fold, $p<0.05$ vs NG ctrl) in HMEC cultured in intHG+hypo and corrected by both T and FA (**Fig. 3a,d**). VEGF was enhanced also in HRP cultured in hypoxic conditions, regardless of glucose concentrations, and normalized by thiamine (**Fig. 3b**). Unexpectedly, VEGF decreased in Müller cells cultured in diabetic-like conditions (**Fig. 3c**).

IntHG, with/without hypoxia, augmented Ang-1 and Ang-2 release by HMEC and Müller cells, but not pericytes (**Fig. 4**). Thiamine normalized Ang-2 production in HMEC (**Fig. 4d**), and FA reduced Ang-1 release in MIO-M1, when added to intHG+hypo (**Fig. 4c**). Surprisingly, FA enhanced both Ang-1 and Ang-2 production by HMEC (**Fig. 4a,d**).

PDGF-B, responsible for vessel stabilization, was unchanged in all cell types in diabetic-like conditions (**Fig. 4g,h,i**), but FA increased its production in HMEC in all conditions and in MIO-M1 in intHG+hypo (**Fig. 4g,i**). MCP-1 release, a marker of inflammation, was enhanced in HRP and Müller cells in intHG+hypo and normalized by FA (**Fig. 4j,k**). MCP-1 release by HMEC was undetectable with this method.

The scheme in **Fig. 5** shows a summary of the possible interactions among all these different molecules involved in diabetes-induced damage.

Tube formation assay

To evaluate the capability of our system to produce neo-vascularization in response to diabetic-like stimuli, we co-cultured HMECs and HRPs in Matrigel. Vessel-like formation after 24h was increased by 15%, when cells were pre-cultured in intHG ($p<0.05$ vs NG ctrl) (**Fig. 6**), and thiamine reverted this effect. However, vessel formation was strongly decreased in cells submitted to hypoxic treatment, probably because HMEC were less viable following this treatment, as well as for cells treated with FA.

Discussion

Finding non-invasive pharmacological treatments to prevent or arrest the development of DR is a primary goal. We studied the effects of two molecules that have been addressed as potential treatments, thiamine and fenofibrate, on cell models mimicking the iBRB, in the diabetic retinal microenvironment.

The iBRB is involved in the early phases of DR. Endothelial cells are directly exposed to the hyperglycemic insult from the blood flow, while pericytes play a key-role in the maintenance of the iBRB: their loss and migration are probably the *primum movens* of the disease, followed by capillary closure, hypoxia and generation of angiogenic molecules. Loss of pericyte control on endothelial proliferation leads to new vessel sprouting and, eventually, angiogenesis [2]. Müller cells, spread across the whole retina and linking its vascular and neural sides [3], are responsible for the uptake of neurotransmitters and nutrients and, in diabetes, can contribute to neuronal dysfunction, inflammation and angiogenesis by releasing cytokines, chemokines and growth factors [3].

The *in vitro* model of the iBRB we re-created using species-specific cells only is meant as close as possible to the human retinal microenvironment in diabetic-like conditions: pericytes and Müller cells are of retinal origin, while HMECs, though derma-derived, are largely used as models for the study of various diseases, including DR [19]. We chose intermittent high glucose conditions for our experimental setting, because HRP, ECs and Müller cells are more sensitive to intermittent than stable high glucose [16, 20, 21] and, in real life, diabetic patients are continuously subjected to glucose fluctuations. It has also been postulated that glycemic excursions may cause retinal neurodegeneration in type 1 diabetes [22].

Our results show increased expression of integrin $\alpha v\beta 3$, integrin subunit β1 and ICAM-1 in ECs exposed to fluctuating high glucose and hypoxia. β1 and ICAM-1 were increased also in pericytes, but not in Müller cells. Thiamine supplementation reduced this overexpression. Cell surface integrins play important roles in the integrity of extracellular matrix (ECM), but also bind and internalize several other molecules [23]. Integrins $\alpha v\beta 3$, $\alpha v\beta 1$ and $\alpha 2\beta 1$ are involved in angiogenesis, the latter through their subunit β1, by promoting cell migration, proliferation and survival of angiogenic ECs [23]. ICAM-1 is mainly expressed by EC and leukocytes, following cytokine stimulation. Leukocytes are involved in the pathogenesis of many retinal diseases, among which DR [24], and their adhesion is regulated by ICAM-1 and MCP-1 [23, 24]. ICAM-1 is also involved in angiogenesis, as VEGF can enhance its expression [24].

Increased MMP2 and MMP9 expression, and the concomitant reduction of TIMP1, as found in our cell models, are consistent with our previous findings showing dysregulation of the MMPs/TIMP1 system in HRPs due to high glucose damage [17], as well as increased MMP2 in the same cells exposed to extracellular vesicles released in diabetic-like conditions [18]. Our present results demonstrate that thiamine supplementation corrects this damage. MMPs are primarily involved in ECM degradation, but they are also able to cleave other non-ECM proteins, including growth factors and cytokines [25]. MMP2 and MMP9 enhance angiogenesis, modulating several mechanisms, such as VEGF [25], and hyperglycemia induces MMP upregulation via oxidative stress and AGE formation [25]. MMP2 and MMP9 are expressed also by Müller cells [26]: cytokines induce upregulation of MMP9 [26] and there is evidence of increased MMPs in high glucose conditions [21]. Increased levels of MMP2 and MMP9 were found in the retina and vitreous of DR patients [27]. However, we could not detect any modulation of MMP2 and 9 in Müller cells, apart from a decrease following thiamine supplementation.

The hypoxic microenvironment characteristic of DR induces the nuclear translocation of HIF-1α, which binds to inducible target genes, increasing expression of angiogenic proteins, such as VEGF [28]. In a previous paper, we demonstrated that HIF-1α expression is increased in circulating extracellular vesicles from DR subjects [29]. VEGF is secreted ubiquitously inside the retina and its expression is also modulated by high glucose [28]. It induces both increased iBRB permeability and angiogenesis, two major events of DR,

and its expression is augmented in the vitreous and aqueous humor of DR patients [30]. Consistently with previous findings, our results show a major increase of HIF-1α in ECs cultivated in hyperglycemic and hypoxic conditions, with a consequent rise of VEGF. VEGF was also upregulated in pericytes by hypoxia, while Müller cells were not affected. Addition of thiamine was again able to normalize enhanced VEGF and HIF-1α release.

Ang-1 and Ang-2 are strictly involved in the pathological mechanisms at the basis of DR, since they partly regulate pericyte recruitment and endothelial cell survival [31]. In our experiments, we found upregulation of both of them in diabetic-like conditions in ECs and Müller cells, but not pericytes. Thiamine was able to normalize Ang-2 in ECs and Ang-1 in Müller cells, while FA increased both Ang-1 and Ang-2 release by ECs. Ang-1 has a dual role in angiogenesis: as an angiogenic factor, it plays a key-role in pericyte recruitment and vessel stabilization [32], but it has also been shown to inhibit tumor growth [31] and abnormal angiogenesis during DR [31]. Ang-2 has been long considered as an antagonist of Ang-1, as it binds the same receptor (Tie2), with a role in vascular disruption. However, it has also agonist functions, depending on the context [33]. Ang-2 expression is increased in vascular cells by hyperglycemia [31], inflammatory mediators [33], hypoxia and VEGF [34], and its role in pericyte dropout during DR has been demonstrated [31]. Müller cells showed increased Ang-2 expression under hyperglycemic conditions *in vitro* [31]. Interestingly, Ang-2 can exert angiogenic functions also independently of Tie2, by binding directly to integrins, especially the β1 subunit [35].

PDGF-B/PDGF-receptor β signaling is crucial for the proliferation, migration and recruitment of pericytes to the microvasculature [36]. Hyperglycemia reduces PDGF-B/PDGFR-β signaling in animal models of DR, leading to pericyte apoptosis and pro-inflammatory responses, and inducing release of MCP-1 from endothelium [37]. MCP-1 acts as a potent inflammatory factor inside the retina, released mostly by Müller cells, its levels correlating with VEGF production, changes in BRB permeability, leukocyte recruitment, and induction of superoxide from monocytes/macrophages [25]. Its expression is increased in patients with ocular diseases, such as DR [30]. Consistently, we found a decrease in PDGF-B in ECs cultured in fluctuating glucose concentrations, corrected by thiamine supplementation, and increased MCP-1 release by pericytes and Müller cells, normalized by FA addition. MCP-1 was undetectable in ECs.

The relationship among all the molecules investigated in this study and involved in diabetes-induced damage is summarized in Fig. 5.

Thiamine is beneficial in preventing damage caused by hyperglycemia *in vitro* and in animal models [7, 38, 39]. This effect is obtained mainly through its anti-oxidant properties, as it reduces increased ROS production, by acting on the four metabolic branches of the unifying mechanism proposed to mediate glucose-induced injury [5, 40, 41]. Thiamine deficiency leads to a pseudo-hypoxic state, with accumulation of VEGF and HIF-1α [42]. Thiamine acts also indirectly on ECM impairment in hyperglycemic conditions, which causes pericyte loss [43]. In diabetic animals, thiamine reduced diabetic nephropathy and DR [5, 6]. Diabetic patients show renal loss of thiamine, causing kidney damage [4]. Recently, two SNPs in SLC19A3 gene encoding for THTR2 were associated with absent/low DR and nephropathy in long-term type-1 diabetes [44] and primary involvement of THTR2 in thiamine transport inside retinal cells, with a role in hyperglycemic-induced damage and impaired thiamine availability was hypothesized [7]. Our results show a generalized beneficial effects of thiamine supplementation on all the metabolic pathways addressed, possibly achieved by switching off the activating signal cascade initiated by free radical accumulation.

Fenofibrate is a peroxisome proliferator-activated receptor alpha (PPARα) agonist. Two clinical trials, the FIELD study [8] and the ACCORD-Eye study [9], showed that it reduces the risk of progression of DR. However, its mechanisms of action in DR remain unclear, though almost certainly independent of its lipid-lowering effects [10]. Increased blood vessel permeability and retinal vascular leakage in diabetic animal models were ameliorated by PPARα overexpression and administration of oral FA [11], suggesting a role as

an anti-inflammatory agent [12]. Moreover, FA prevents the disruption of the outer BRB [45] and could also have anti-angiogenic effects, reducing expression of VEGF receptor-2 in ECs, thus inhibiting neovascularization [13]. Other studies show a negative modulation of VEGF and decreased loss of ECs and pericytes in the presence of FA [46]. However, in other districts, FA was shown to have opposite effects, restoring impaired VEGF production and migration in EC [47].
In our study, FA supplementation reduced integrin β1, MMP9, Ang-1 and MCP-1 expression in pericytes in diabetic-like conditions, as well as Ang-1 and MCP-1 in Müller cells. However, surprisingly, it exhibited controversial effects on ECs, slowing down their proliferation and increasing Ang-1/Ang-2 release, while conversely lowering VEGF/HIF-1α expression. This reflects also on the tube formation assay, where cells exposed to FA form very few new tubes in comparison with physiological control, with a rather suffering aspect. Since we have chosen the same FA concentration found beneficial in previous reports on retinal cells [10, 13, 45], these effects could rather be due to our experimental setting, that implies 8-day exposure, instead of the 48h treatment used by most of the other works, and should be taken into account when extrapolating data from cell cultures exposed for very short periods to different drugs.

Conclusions

Taken together, our results show that microvascular cells of the iBRB are more affected than Müller cells by the hyperglycemic and hypoxic insult characteristic of the diabetic retinal microenvironment. Müller cells are known to be very resistant elements, as they produce several self-protective factors and begin to die off later than pericytes and ECs in the natural history of DR [3]. FA shows a controversial behavior, potentially positive on Müller cells and HRP, but possibly damaging on endothelium, while thiamine confirms once more to be effective in reducing diabetes-induced retinal damage.

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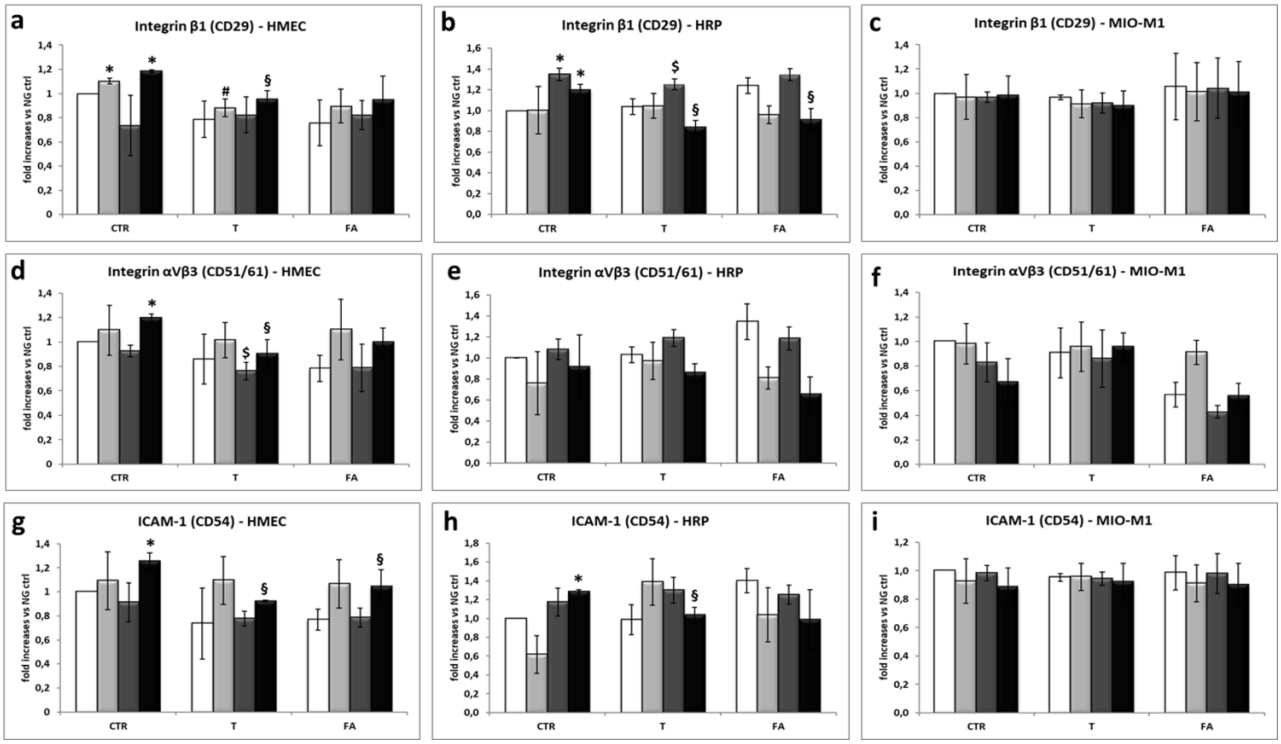

Fig. 1 Expression of the adhesion molecules integrin β1 (CD29) (a, b, c), **integrin αVβ3 (CD51/61)** (d, e, f) and **ICAM-1 (CD54)** (g, h, i) in HMECs (a, d, g), HRPs (b, e, h) and Müller cells (c, f, i), as detected by FACS analysis. *White bars*: NG, *light grey bars*: NG+hypo, *dark grey bars*: intHG, *black bars*: intHG+hypo. CTR: control, T: thiamine, FA: fenofibrate. N=5, * p<0.05 vs NG CTR, # p<0.05 vs NG+hypo CTR, $ p<0.05 vs intHG CTR § p<0.05 vs intHG+hypo CTR

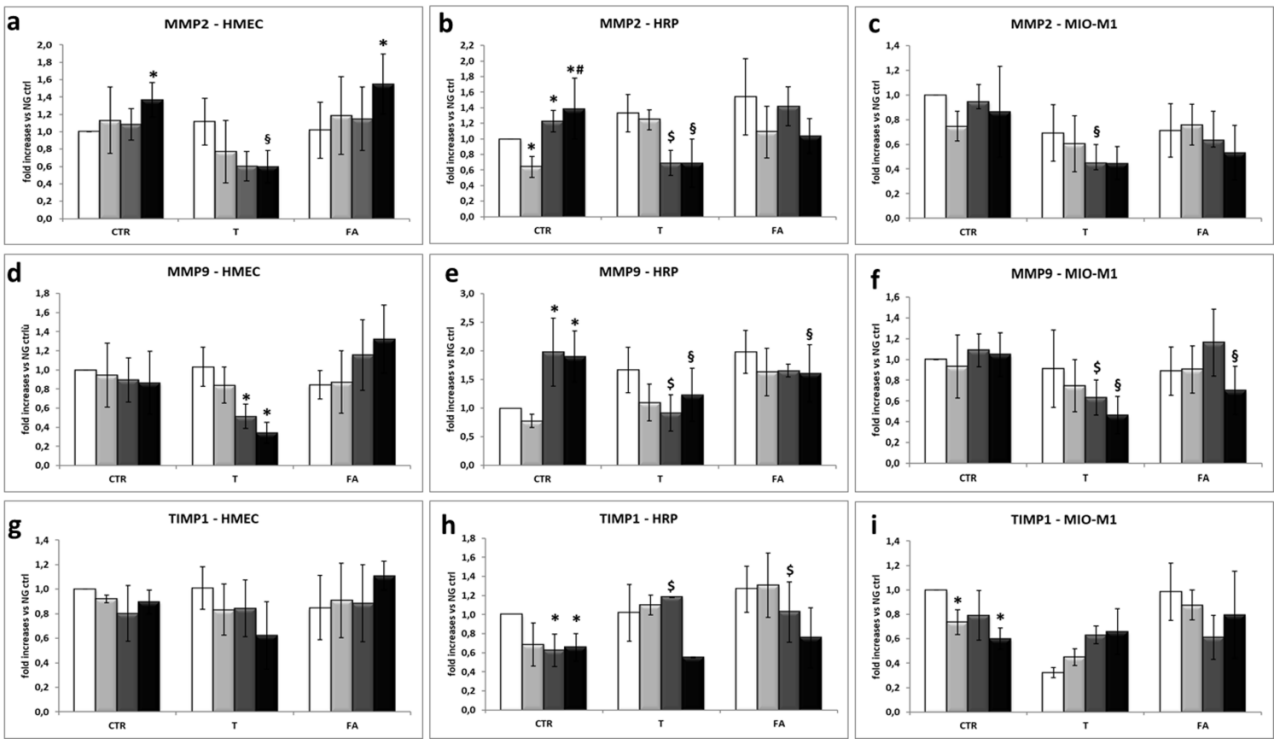

Fig. 2 Expression of metalloproteases MMP2 (a, b, c), **MMP9** (d, e, f) and **their inhibitor TIMP1** (g, h, i) in HMECs (a, d, g), HRPs (b, e, h) and Müller cells (c, f, i), as detected by WB in cell lysates. *White bars*: NG, *light grey bars*: NG+hypo, *dark grey bars*: intHG, *black bars*: intHG+hypo. CTR: control, T: thiamine, FA: fenofibrate. N=5, * p<0.05 vs NG CTR, $ p<0.05 vs intHG CTR § p<0.05 vs intHG+hypo CTR

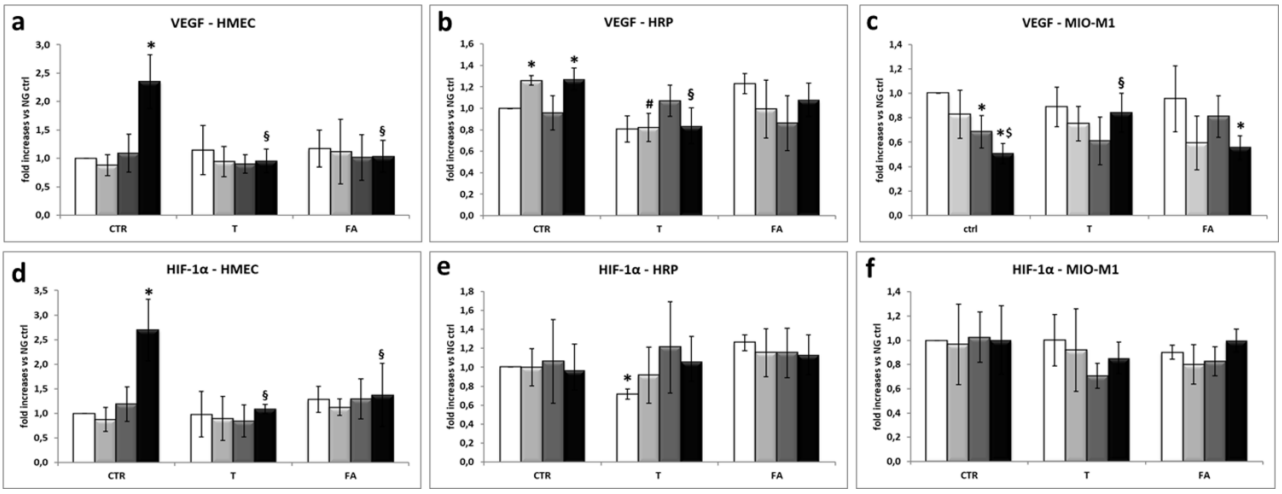

Fig. 3 VEGF (a, b, c) and **HIF-1α** (d, e, f) **expression** in HMECs (a, d), HRPs (b, e) and Müller cells (c, f), as detected by WB in cell lysates. *White bars*: NG, *light grey bars*: NG+hypo, *dark grey bars*: intHG, *black bars*: intHG+hypo. CTR: control, T: thiamine, FA: fenofibrate. N=5, * p<0.05 vs NG CTR, # p<0.05 vs NG+hypo CTR, $ p<0.05 vs intHG CTR § p<0.05 vs intHG+hypo CTR

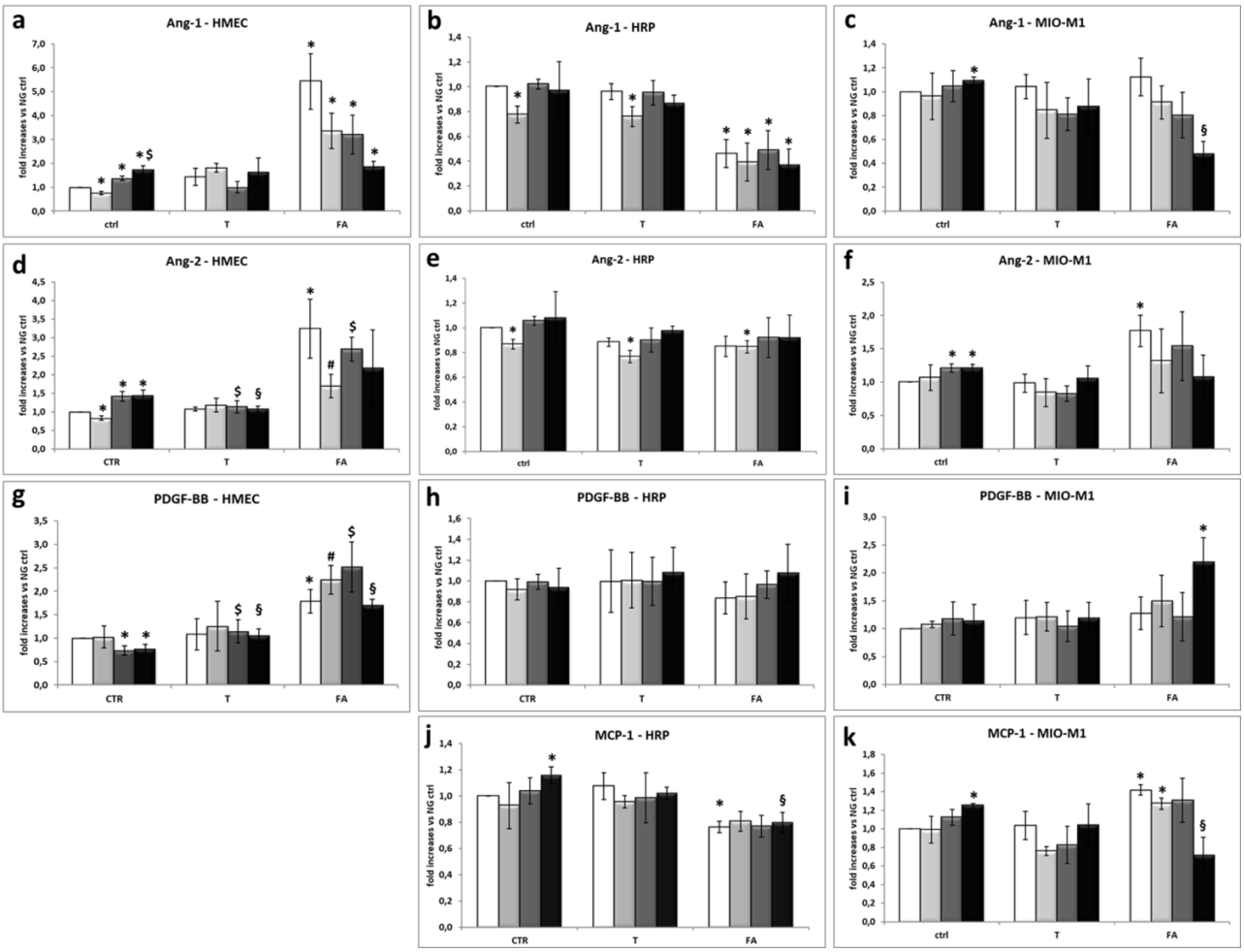

Fig. 4 Ang-1 (a, b, c) , **Ang-2** (d, e, f), **PDGF-BB** (g, h, i) and **MCP-1** (j, k) **release** by HMECs (a, d, g), HRPs (b, e, h, j) and Müller cells (c, f, i, k), as detected by ELISA in cell culture supernatants. MCP-1 release by HMEC was undetectable with this method. *White bars*: NG, *light grey bars*: NG+hypo, *dark grey bars*: intHG, *black bars*: intHG+hypo. CTR: control, T: thiamine, FA: fenofibrate. N=5, * p<0.05 vs NG CTR, # p<0.05 vs NG+hypo CTR, $ p<0.05 vs intHG CTR § p<0.05 vs intHG+hypo CTR

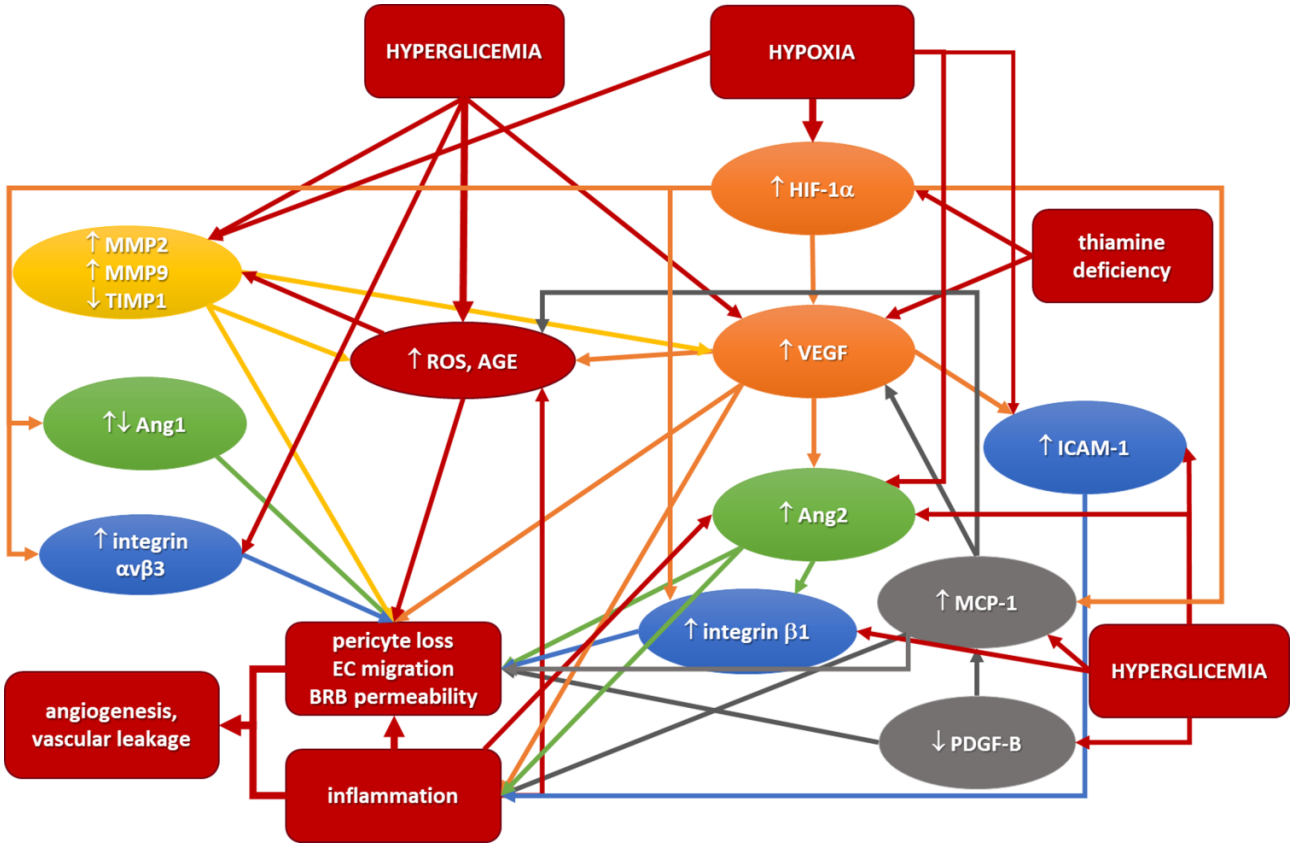

Fig. 5 Interactions among the different molecules involved in diabetes-induced damage, as described in the literature and examined in this work. Hyperglycemia and hypoxia directly, or indirectly through increased ROS/HIF-1α production, enhance expression of cell surface molecules (integrin αvβ3, integrin subunit β1, ICAM-1, MMP2/9), involved in pericyte loss and angiogenesis. The hypoxic microenvironment induces the nuclear translocation of HIF-1α, which binds to inducible target genes, modulating the expression of VEGF, integrins, MCP-1 and Ang-1. VEGF, the main actor of retinal angiogenesis, has direct effects on BRB permeability, pericyte loss and inflammation, but also stimulates Ang-2 and ICAM-1 expression. Ang-1/2 regulate pericyte recruitment and EC survival and their increase in hyperglycemic and hypoxic conditions correlates with enhanced angiogenesis and inflammation. PDGF-B/PDGF-receptor β signaling is crucial for the recruitment of pericytes and vessel stabilization: hyperglycemia reduces PDGF-B/PDGFR-β signaling leading to pericyte apoptosis, and inducing release of MCP-1, a potent inflammatory factor whose levels correlate with VEGF production and changes in BRB permeability. Thiamine deficiency leads to a pseudo-hypoxic state, with accumulation of VEGF and HIF-1α.

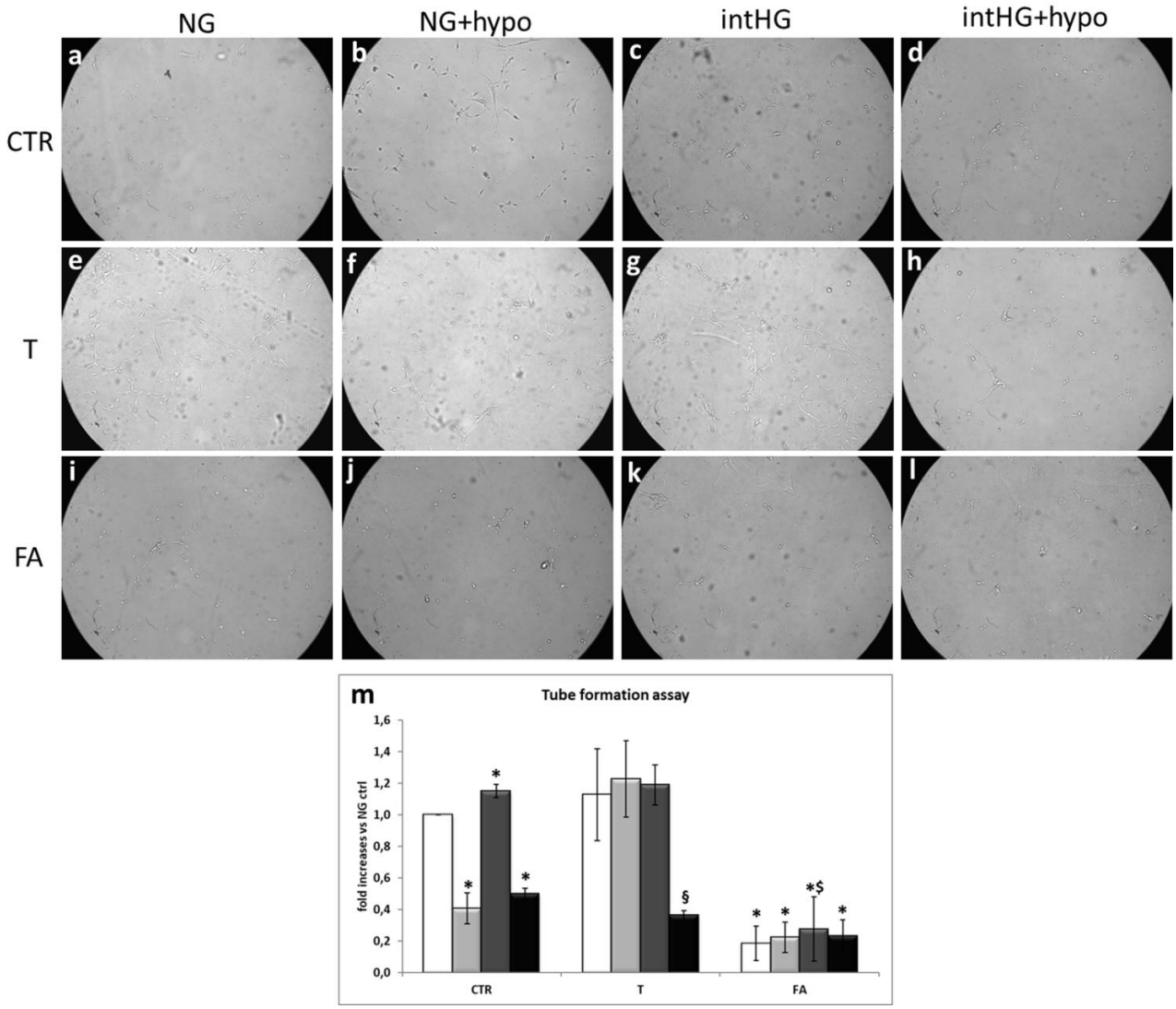

Fig. 6 Tube formation by HMECs and HRPs co-cultured in Matrigel. **a-l**) representative images of one field per each case. **a, b, c, d**: CTRL; **e, f, g, h**: thiamine supplementation**; i, j, k, l**: FA supplementation. **a, e, i**: NG; **b, f, j**: NG+hypo; **c, g, k**: intHG, **d, h, l:** intHG+ hypo. **m**) mean of 5 different experiments. *White bars*: NG, *light grey bars*: NG+hypo, *dark grey bars*: intHG, *black bars*: intHG+hypo. CTR: control, T: thiamine, FA: fenofibrate. * p<0.05 vs NG CTR, # p<0.05 vs NG+hypo CTR, $ p<0.05 vs intHG CTR § p<0.05 vs intHG+hypo CTR