

The $PKC\beta/HuR/VEGF$ pathway in diabetic retinopathy

M. Amadio, C. Bucolo, G.M. Leggio, F. Drago, S. Govoni, A. Pascale

► To cite this version:

M. Amadio, C. Bucolo, G.M. Leggio, F. Drago, S. Govoni, et al.. The $\rm PKC\beta/HuR/VEGF$ pathway in diabetic retinopathy. Biochemical Pharmacology, 2010, 80 (8), pp.1230. 10.1016/j.bcp.2010.06.033 . hal-00618194

HAL Id: hal-00618194 https://hal.science/hal-00618194

Submitted on 1 Sep 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: The PKCβ/HuR/VEGF pathway in diabetic retinopathy

Authors: M. Amadio, C. Bucolo, G.M. Leggio, F. Drago, S. Govoni, A. Pascale

PII:	S0006-2952(10)00467-3	
DOI:	doi:10.1016/j.bcp.2010.06.033	
Reference:	BCP 10614	
To appear in:	BCP	
Received date:	28-4-2010	
Revised date:	21-6-2010	
Accepted date:	21-6-2010	



Please cite this article as: Amadio M, Bucolo C, Leggio GM, Drago F, Govoni S, Pascale A, The PKCβ/HuR/VEGF pathway in diabetic retinopathy, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2010.06.033

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Category: Metabolic Disorders and Endocrinology

The PKC \beta/HuR/VEGF pathway in diabetic retinopathy

M. Amadio^{1,*}, C. Bucolo^{2,*§},

G.M. Leggio², F. Drago², S. Govoni¹, A. Pascale¹

¹Department of Experimental and Applied Pharmacology, Centre of Excellence in Applied Biology, University of Pavia, Italy; ²Department of Experimental and Clinical Pharmacology, University of Catania, Italy.

*These two authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

[§]Corresponding author: Claudio Bucolo, Department of Experimental and Clinical Pharmacology, School of Medicine, University of Catania, Viale A. Doria 6, 95125 Catania, ITALY.

Tel. +39 095 7384225; Fax +39 095 7384236

Email: claudio.bucolo@unict.it

WORD COUNT OF THE ABSTRACT: 184

WORD COUNT OF THE MAIN TEXT: 4301

ABSTRACT

We investigated whether the diabetes-related PKC β activation affects VEGF expression through the mRNA-stabilizing human *Embryonic Lethal Abnormal Vision* (ELAV) protein, HuR, in the retina of streptozotocin (STZ)-induced diabetic rats. Diabetes was induced in rats by STZ injection. Retinal tissues were processed to detect PKC β I, PKC β II, VEGF and HuR contents, as well as HuR phosphorylation. Immunoprecipitation coupled to RT-PCR was employed to evaluate HuR binding to VEGF mRNA in RiboNucleoProteic (RNP) complexes. Statistical analysis was performed by ANOVA followed by an appropriate *post hoc* comparison test. Following experimental diabetes PKC β I and PKC β II levels were increased compared to sham; there was also a PKC-mediated phosphorylation/activation of HuR. These effects were blunted by the *in vivo* co-administration of a selective PKC β inhibitor. A specific binding between the HuR protein and the VEGF mRNA was also detected. The PKC β /HuR activation was accompanied by enhanced VEGF protein expression that was, again, blunted by the PKC β inhibitor. These findings first demonstrate the activation, in the retina, of the PKC β /HuR/VEGF pathway following experimental diabetes and disclose a new potential pharmacological target to counteract pathologies implicating VEGF deregulation, such as diabetic retinopathy.

Keywords: Diabetes; Embryonic Lethal Abnormal Vision protein; PKC; VEGF; Retina.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder that results due to a failure of the body to produce the hormone insulin and/or an inability of the body to respond adequately to circulating insulin. Type 1 diabetes, previously referred to as insulin-dependent diabetes mellitus (IDDM), occurs most commonly in children or young adults and constitutes 5-10% of the diagnosed diabetes patient population. Type 2 diabetes, previously known as adult-onset diabetes or non-insulindependent diabetes mellitus (NIDDM), accounts for 90-95% of diagnosed diabetes cases globally and typically develops in middle-aged adults. Both type 1 and type 2 patients develop retinopathy, in particular almost everyone with type 1 diabetes will develop retinopathy over a 15- to 20-year period and greater than 60% of type 2 diabetes patients will have retinopathy [1]. Diabetic retinopathy is the leading cause of adult visual impairment among people in developed countries, andit can be classified into two stages: nonproliferative and proliferative. The earliest visible signs in nonproliferative diabetic retinopathy are microaneurysms and retinal hemorrhages. Progressive capillary nonperfusion is accompanied by the development of cotton-wool spots, venous beading, and intraretinal microvascular abnormalities. Proliferative diabetic retinopathy occurs with further retinal ischemia and is characterized by the growth of new blood vessels on the surface of the retina or the optic disc. These abnormal vessels may bleed, resulting in vitreous hemorrhage, subsequent fibrosis, and tractional retinal detachment. Diabetic macular edema, which can occur at any stage of diabetic retinopathy, is characterized by increased vascular permeability and the deposition of hard exudates at the central retina and represents the principal cause of vision loss in persons with diabetes.

Unfortunately, medical prevention and treatment of diabetic retinopathy is currently based on optimized control of blood glucose and blood pressure, but cannot rely on specific pharmacologic drugs [2]. Typical early changes of this pathology, in the retina, are the thickening of

the basement membrane, hyper-permeability and formation of microaneurysms. These functional alterations are followed by microvascular occlusions leading to a progressive retinal ischemia that induces the release of the Vascular Endothelial Growth Factor (VEGF), also known as vascular permeability factor. VEGF is a potent mitogen for endothelial cells that triggers proliferation, migration and tube formation leading to growth of new blood vessels that, however, in the diabetic retina are fragile and may break. VEGF (also called VEGF-A) is the major regulator of physiological and pathological angiogenesis [3], and belongs to a family that includes placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E [4,5]. In 1948 Michaelson first suggested the implication of a generic angiogenic factor in the neovascularization process occurring in retinopathy [5]; nowadays several studies stress that a key role in the development of diabetic retinopathy is indeed played by VEGF [6,7]. The expression of VEGF in diabetic retina can be regulated by different pathways, , such as Rho/Rho Kinase, ERK1/2, and Protein Kinase C (PKC) [8-10]. PKC is a family of at least 10 serine-threonine kinases ubiquitously expressed and able to participate in multiple cellular functions [11,12]. Among the different PKC isoenzymes, the beta $(PKC\beta)$ one seems to be preferentially activated in the retina, thus possibly contributing to the early stages of retinopathy [13,16], although other PKC isoenzymes are also activated such as alpha and delta [16,17]. To date no findings are available on how PKC may exert its modulation on VEGF expression. However, a clue comes from our previous in vitro data suggesting the existence, in retinal pericytes, of a new molecular cascade involving PKCβ, the mRNA-Binding Protein (RBP) HuR and VEGF [18]. In that paper, we showed the presence of a new molecular cascade operating in the cytoskeleton of retinal bovine pericytes controlling VEGF expression, although it should be stressed that the study [16] was carried out in a milieu quite different from an in vivo system. HuR is the ubiquitously expressed member of the ELAV (Embryonic Lethal Abnormal Vision) family, highly conserved RBPs which act post-transcriptionally as positive regulators of gene expression [19,20]. The relevance of post-transcriptional events in the regulation of VEGF production is

emphasized by the observation that while in normal conditions VEGF mRNA is extremely labile, its half-life can increase during hypoxia, such as following retinal capillaries occlusion, by 2-3 fold due to the stabilizing effect of HuR [21,22]. Moreover, Tang et al. [23] demonstrated that acute ischemia induces a rapid binding of HuR to the VEGF mRNA leading to increased levels of the corresponding VEGF protein. HuR seems to sterically protect VEGF mRNA from ribonucleases [24], by masking specific instability signals within the VEGF mRNA sequence itself, thus, on the one hand, reducing its decay rate and, on the other, enhancing its translation. The purpose of the present work was to investigate whether the PKCβ/HuR/VEGF pathway, previously described *in vitro*, is operant also *in vivo* in the retina and altered by diabetes.

2. Materials and methods

2.1. Animals and reagents

Male Sprague-Dawley rats weighing approximately 200 g were obtained from Charles River (Calco, Italy). All the animals were treated according to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were fed on standard laboratory food and were allowed free access to water in an air-conditioned room with a 12-h light/12-h dark cycle. Final group sizes for all measurements were n= 6-9 except as noted. Streptozotocin (STZ) and phenyl-methyl sulfonyl fluoride were purchased from Sigma-Aldrich (Milan, Italy). LY379196, a bisindolylmaleimide derivative of staurosporine that shows high selectivity for the β isoforms of PKC [25], was a kind gift of Eli-Lilly, and was used as tool in all experiments. All the other reagents were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise stated.

2.2. Induction of diabetes

STZ destroys pancreatic island β cells and is used to induce experimental diabetes in rodents. STZ-induced diabetic rats show the same characteristics of non-proliferative diabetic

retinopathy seen in humans including blood vessel dilation, capillary degeneration, increased leukostasis, and increased vascular permeability resulting from breakdown of the blood-retinal barrier, loss of endothelial cells and pericytes from capillary beds, microaneurysm formation, changes in retinal electrophysiological activity, decreases in the thickness of the inner plexiform and inner nuclear layers of the retina, and a decrease in retinal ganglion cell density that appears to be due to apoptotic cell death. After 12 h of fasting, the animals received a single 60-mg/kg intravenous (i.v.) injection of STZ in 10 mM sodium citrate buffer, pH 4.5 (1 mL/kg dose volume). Control (sham, non-diabetic) animals were fasted and received citrate buffer alone. After 24 h, animals with blood glucose levels >250 mg/dl were considered diabetic, and randomly divided into groups of ten animals each. The diabetic state was confirmed by evaluating glycemia daily through a blood glucose meter (Accu-Check Active[®], Roche Diagnostic, Milan, Italy). The PKCβ inhibitor was given at 1 mg/kg (i.p.) per day. All the experiments were performed 10 days after the induction of diabetes. In our experiments, no supplemental insulin was administered to prevent weight loss.

2.3. Preparation of the samples and western blotting

Rat retinae were collected 10 days after STZ; the tissue was homogenized in Buffer A [20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 50 mM mercaptoethanol, 0.32 mM sucrose, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) at the dilution suggested by the manufacturer using a teflon/glass homogenizer. Proteins were measured according to Bradford's method using bovine albumine as internal standard. Proteins were diluted in 2X SDS protein gel loading solution, boiled for 5 min, separated on 12% SDS- PAGE and processed as previously described [26]. The anti-PKCβI mouse monoclonal antibody (Santa Cruz, CA, USA) was diluted at 1:500, the anti-PKCβII rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:300, the anti-HuR mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000, the anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:150 and the anti-

 α -tubulin rat monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) at 1:1000. The nitrocellulose membrane signals were detected by chemiluminescence. Experiments were performed at least three times for each cell preparation; the same membranes were re-probed with α -tubulin antibody and used to normalize the data. Statistical analysis of western blot data was performed on the densitometric values obtained with the NIH image software 1.61 (downloadable at <u>http://rsb.info.nih.gov/nih-image</u>).

2.4. Preparation of mRNP (RiboNucleoProteic complexes)

The retinal tissues were homogenized in ice-cold polysome lysis buffer [containing 100 mM KCl, 5 mM MgCl₂, 10 mM Hepes (pH 7), 0.5% Nonidet P-40 and 1 mM DTT (Dithiothreitol)] supplemented with RNase and protease inhibitors, mixed by pipetting several times, placed on ice for 10 min, frozen and stored at -80° C until use. The homogenates were thawed and centrifuged in a tabletop microcentrifuge at 14,000 x g for 10 min at 4° C. The supernatant was removed and centrifuged again in the same manner. The final supernatant, representing a fraction particularly enriched in mRNAs and proteins (called mRNP), was stored at -20° C until use for immunoprecipitation experiments.

2.5. Immunoprecipitation

Samples (total lysates or mRNP) were processed according to a previously published protocol with minor modifications [27]. Briefly, immunoprecipitation was performed at room temperature for 2 h using 1µg of anti-HuR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) per 50µg of proteins diluted with an equal volume of 2x Immunoprecipitation Buffer [2% Triton X-100, 300mM NaCl, 20mM Tris-HCl (pH 7.4), 2mM EDTA, 2mM EGTA, protease inhibitor cocktail and an RNAase inhibitor] in presence of 50µl of protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA USA) previously blocked with 5% BSA in PBS. The samples were finally subjected to either western blotting or RNA extraction. The western blottings on the

immunoprecipitated proteins were performed using the mouse antibodies anti-phosphothreonine diluted at 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA USA) and anti-phosphoserine diluted at 1:500 (Sigma-Aldrich, Milan, Italy). The negative control was obtained under identical conditions, but in the presence of an irrelevant antibody (IRR) with the same isotype of the specific immunoprecipitating antibody. Small aliquots of the immunoprecipitation mixes were collected from each sample and used as "input signals" (I.S.) to normalize the western blotting or the Real Time-PCR (RT-PCR) data. The Positive Control (PC) was represented by a total retinal homogenate obtained from additional control rats, homogenized in Buffer A, and processed as described in the section 2.3.

2.6. Quantitative Real Time-PCR

RNA was extracted from total homogenates and immunoprecipitated pellets using RNeasy Micro Kit (Qiagen, Milan, Italy). The reverse transcription was performed following standard procedures. PCR amplifications were carried out using the Lightcycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) in the presence of FastStart SYBR Green master mix (Roche Molecular Biochemicals, Mannheim, Germany) and with the specific primers for VEGF: 5'-TCACCAAAGCCAGCACATAG-3'; 5'-Forward: Reverse: AACAAGGCTCACAGTGATTTTCT-3'. GAPDH (Glyceraldehyde 3-Phosphate The Dehydrogenase) mRNA, whose primers are: 5'-CAGCAAGGATACTGAGAGCAAG-3' (upstream), and 5'-GGATGGAATTGTGAGGGAGA-3' (downstream), was chosen as reference [28] on which VEGF values were normalized and to check the specificity of the HuR binding to VEGF. The quantification (expressed in Cycle threshold, Ct) of VEGF mRNA in the total homogenates and immunoprecipitated pellets was performed as previously published [29]. Moreover, as concerns the immunoprecipitated pellets the amplified products were also run in an agarose gel.

2.7. ELISA assay for VEGF

Retinal tissues were homogenized in 100 μ l of solution consisting of 20 mM imidazole hydrochloride, 100 mM KCl, 1 mM MgCl, 1 mM EGTA, 1% Triton, 10 mM NaF, 1 mM sodium molybdinate, and 1 mM EDTA. The solution was supplemented with a cocktail of protease inhibitors (Complete, Roche, Basel, Switzerland) before use. The samples were cleared *via* centrifugation for 10 min at 10,000 x g and assessed for protein concentration with the bicinchoninic acid (BCA) assay (Mini BCA kit; Pierce Scientific, Rockford, IL, USA). The VEGF protein levels were estimated with the respective ELISA kit (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. All the measurements were performed in duplicate. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

2.8. Data analysis

For statistical analysis the GraphPad Instat statistical package (version 3.05 GraphPad software, San Diego, CA, USA) was used. The data were analyzed by analysis of variance (ANOVA) followed, when significant, by an appropriate *post hoc* comparison test. Differences were considered statistically significant when p values ≤ 0.05 .

3. Results

3.1. Glycemia and Body Weight

Induction of diabetes with STZ is associated with hyperglycemia and weight loss. After ten days from STZ administration, blood glucose values in diabetic rats were significantly higher than the corresponding values in non-diabetic animals (408 ± 23 and 99 ± 14 mg/dl respectively; see Table 1). A decrease in body weight of diabetic rats was observed, as also reported by others [30]. The PKC β selective inhibitor LY379196 did not interfere with glycemia and body weight (Table 1), this finding is in agreement with literature data showing that the repeated administration of a

specific PKC β inhibitor, closely related to LY379196, does not induce changes in body weight or glucose levels in rats [31].

3.2. Upregulation of PKC β and phosphorylation of HuR in the retina of diabetic rats

Considering that PKC signal transduction is enhanced in the hyperglycemic state [16], and that PKC_β isoforms are especially implicated in diabetic retinopathy, we first evaluated the protein content of both PKCBI and BII in retinal tissues from STZ-induced diabetic rats. As shown in Fig. 1, both PKCBI and BII isotypes increased following streptozotocin with respect to sham animals (+160%, Fig. 1A; +113%, Fig. 1B). This up-regulation is possibly related to the activated PKCB that is able to stimulate its own transcription leading to an enhanced production of the corresponding protein [32]. Indeed, this effect was counteracted by the *in vivo* co-administration of the selective PKCβ inhibitor LY379196 (Fig. 1A and 1B). We previously found *in vitro* evidence that PKCβ and HuR belong to the same molecular cascade [18]; it was then of great interest to verify the existence of this pathway in vivo exploring HuR protein content and its PKC-dependent phosphorylation (as an indirect index of kinase activity). As reported in Fig. 2, HuR protein levels increased in STZ-treated rats (+62% vs. control, Fig. 2A) and, more interestingly, this was accompanied by a higher phosphorylation in serine residues (+209% vs. control, Fig. 2B). This last finding is in line with the high number of serine residues within the aminoacidic sequence of HuR and with our previous results in retinal pericytes [18]. The effects on the HuR protein were specifically related to PKCβ since they were blunted by the selective inhibitor LY379196 (Fig. 2A and 2B).

3.3. Effect of the PKC\u03b3/HuR cascade activation on VEGF

We previously demonstrated that in retinal bovine pericytes PKC β activation is able, through the RNA-binding protein HuR, to increase VEGF protein levels [18]. Given the triggering of the PKC β /HuR cascade in retinal tissues from diabetic rats, we investigated its consequences on

VEGF expression. We first evaluated the content of VEGF mRNA in retinal tissues from control and STZ-induced diabetic rats to explore whether diabetes influences VEGF transcription. The results indicate that total VEGF mRNA levels do not change following diabetes, moreover they are not even affected by the concomitant treatment with LY379196 (mean of Ct values: CON= 28.8 + 0.86; STZ= 28.5 ± 0.84 ; STZ+ LY379196= 28.7 ± 1.02 ; n=6; see also Fig. 3 A and B). These data suggest that in our model VEGF expression is not modified at the transcriptional level and prompted us to investigate whether this gene undergoes a post-transcriptional regulation. We thus initially examined the existence of a specific binding between the HuR protein and VEGF mRNA in the retina. For this we isolated the mRNPs, performed immunoprecipitation with the anti-HuR antibody and finally detected the VEGF mRNA by RT-PCR. Immunoprecipitation with an irrelevant antibody with the same isotype of HuR antibody was used as a negative control. The amplification curves indicate that the amount of VEGF transcript is much higher in the samples immunoprecipitated with the anti-HuR antibody (Fig. 3C). These results are supported by the observation that when the amplified products from RT-PCR were run in an agarose gel, VEGF mRNA is only detectable in the anti-HuR immunoprecipitated samples (Fig. 3D), thus indicating the specificity of the binding between the HuR protein and VEGF mRNA. Additionally, as expected, in these same samples the GAPDH mRNA was almost undetectable (not shown). The RT-PCR analysis on the anti-HuR immunoprecipitated samples from control and treated (STZ and STZ+LY379196) animals indicates that the amount of VEGF mRNA bound to HuR protein is similar in the all retinal tissues, although a non significant slight increase was observed in STZinduced diabetic rats in comparison to sham (STZ vs. CON +12%; STZ+LY379196 vs. CON +3%; n=6). We finally evaluated the effects of diabetes on VEGF translation by western blotting. The results point out that VEGF protein content is significantly increased in STZ-induced diabetic rats (+256% vs. control, Fig. 4A); again, the in vivo co-administration of the selective inhibitor LY379196 restores the VEGF protein levels to normal conditions (Fig. 4A).

3.4. VEGF levels via ELISA

We confirmed the increase of VEGF levels in the retinal tissues also using another technique, the ELISA assay. As shown in Fig. 4B, the retina of diabetic animals showed an 8.4-fold increase in VEGF levels compared with the retina of non-diabetic animals. The *in vivo* co-administration of LY379196 significantly reduced the retinal VEGF detected in diabetic rats (from 5.92 ± 0.24 pg/mg to 1.73 ± 0.35 pg/mg). Noteworthy, other groups demonstrated that vitreous VEGF levels were higher in diabetic rats than in non-diabetic controls. In particular, Kusari et al. [33], Xu et al. [34] and Cohen et al. [35] found that the vitreous of STZ-induced diabetic rats showed a 2-fold, 4-fold and 2.2-fold increase in VEGF levels compared with non-diabetic animals, respectively. In contrast, there are no evidence of plasma VEGF changes in STZ-induced diabetic rats.

4. Discussion

Hyperglycemia is considered the main risk factor responsible for numerous diabetic vascular complications, such as retinopathy. Indeed, it has been documented that an intensive blood glucose management in Type 1 diabetes successfully delays the onset and the progression of retinopathy and other diabetes-related pathologies [36]. Several molecular mechanisms have been used to explain the dysfunctions associated with elevated glucose levels, including the polyol pathway flux, oxidative stress, and non-enzymatic glycation [37]. Within this context, a relevant place is certainly taken by PKC, whose activation is related to many vascular abnormalities [16]. In fact, hyperglycemia induces, through various routes, an enhanced generation of diacylglycerol, [10,13] the physiological activator of PKC, that at the retinal level seems to predominantly stimulate the beta isoform of the kinase [15], although, as already mentioned, other PKC isoenzymes are also activated [16,17]. The crucial involvement of PKC β is further emphasized, on the one hand, by a

dramatic increase in the angiogenic response to retinal ischemia in transgenic mice overexpressing by a selective inhibitor of this kinase.

PKCB and, on the other hand, by a significant decrease in retinal neovascularization in PKCB null mice [38]. Moreover, besides the retina, it has been observed that the lack of PKCB can protect against diabetic-induced dysfunctions and augment VEGF expression in other tissues affected by this pathology [39]. However, in the retina and in other districts there is evidence indicating that diabetes is associated not only with an increase of PKC activation, but also with augmented PKC protein levels [16,40,41]. Our present data are in line with these findings, showing that in the retina, as a consequence of diabetes, there is an increased amount of both PKCBI and PKCBII proteins. As regards the underlying mechanism, it has been suggested [31] that the activated PKCB is able to stimulate its own transcription leading to an increased expression of the corresponding protein (see also Fig. 5). In line with this hypothesis, a recent paper reports an enhanced transcription of the PKCB gene in kidney biopsies of diabetic patients compared to normal controls [42]. In agreement with our results, Liu et al. [32] also showed that the up-regulation of PKCβ protein levels is blunted As previously mentioned, literature data underline the implication of PKC in the positive

control of VEGF expression [10]. It has been shown that the expression of this angiogenic factor is increased by elevated glucose levels, even in early stage of diabetes [43], and prevented by general PKC inhibitors [31,44,45]. In the present study we demonstrate that, in the retina, VEGF protein content increases as a consequence of diabetes and this enhancement is prevented by the in vivo concomitant treatment with a selective inhibitor of PKCB. These findings are in agreement with other studies [43,44] reporting that the inhibition of PKCβ pathway hampers VEGF and some of the VEGF-induced vascular dysfunctions. Within this context, the few clinical trials performed with the PKC_β inhibitor ruboxistaurin (LY333531) show that this inhibitor, even though moderately reduces the incidence of visual loss, seems not to affect the progression of proliferative diabetic retinopathy [45]. This observation suggests the possible implication in the latter of VEGF- and PKC-

independent mechanisms [46,47]. On the other hand, VEGF mRNA bears a characteristic motif found in its 3'-untranslated region, called Adenine uridine-Rich Element (ARE), which generally governs the decay rates of specific mRNAs and represents the docking site for some RBPs [48]. It has been shown that, in different cellular models, PKC activation induces the stabilization of AREbearing mRNAs [19,49]. Specifically, our previous *in vitro* work in retinal bovine pericytes describes a new molecular cascade involving PKCβ, the RBP HuR, and VEGF [18] and supports the key importance of post-transcriptional mechanisms in the modulation of VEGF expression [21-23,50].

The post-transcriptional control is a "housekeeping" phenomenon that modifies the content of a particular protein through a modulation of the half-life and/or the translation of the correspondent mRNA. At this regard, RBPs play a critical role being able to affect the processing of selected transcripts, their transport and localization in the cytoplasm, their stability and translation, thus representing the most important *trans*-acting factors in the control of gene expression [19,20,51]. The best characterized RBPs are the ELAV proteins that in the genomes of vertebrates include neuron-specific members (HuB, HuC and HuD) and the ubiquitously expressed HuR [52].

Studies of retinal VEGF mRNA expression in STZ-induced diabetic rats have produced conflicting results, with VEGF mRNA concentrations reported to be increased [34,53,54], decreased [55,56] or unchanged [57] in diabetic rats compared with levels in control rats. The present findings show that, following diabetes, there is a higher phosphorylation of the HuR protein by PKCβ resulting in the activation of HuR itself that then targets VEGF mRNA, finally leading to an increased amount of the correspondent VEGF protein (Fig. 5). This last finding together with the observation that, in our model, VEGF mRNA shows no changes following diabetes, suggests that VEGF may be here regulated mainly at the translational level rather than at the transcriptional level as reported in literature [7], although we cannot exclude a concomitant reduction in VEGF protein degradation. This could represent a breakthrough regarding the regulation of VEGF gene expression

in diabetes, although more data are needed to strengthen this hypothesis. However, the relevance of the post-transcriptional regulation of VEGF has been previously demonstrated, other than by our group [50], also by others [21,23]. Furthermore, an encouraging support comes from the fact that the molecular pathway described now in vivo in the retina resembles the one previously reported in *vitro* in pericytes [18] or in another cellular model [29] where it is triggered rapidly. In general, specific ELAV post-translational modifications, such as phosphorylation by PKC, can represent the final event of signaling cascades able to influence the different functions of ELAV themselves, resulting in the down-stream increased expression of specific ELAV-target mRNAs [29,58]. Our data indicate that it seems to be the case also for VEGF mRNA that, as previously mentioned, bears ARE motifs. Indeed, we demonstrated that HuR binds VEGF mRNA in RiboNucleoProteic complexes ultimately leading to, following experimental diabetes, a higher amount of the correspondent protein. Once more, the in vivo co-treatment with the selective PKCB inhibitor LY379196 [25] prevents HuR phosphorylation and reinstates VEGF protein levels to normal levels, further stressing the idea of a succession of interconnected molecular steps starting from PKC. In agreement with our hypothesis, it has been recently shown [59] that the expression of VEGF significantly decreased in human renal cell carcinoma transfected with siRNA to knockdown HuR.

In the present study we focused on VEGF to confirm *in vivo* the pathway previously described in pericytes, however, we cannot exclude the involvement of other mRNAs that are targeted by HuR [60,61] and involved in angiogenesis as well, such as $TNF\alpha$ [62]. Even though we demonstrated the involvement of isoform beta of PKC, we cannot rule out the implication of other isoforms such as alpha and delta. Indeed, Kim et al. [63] demonstrated that PKC delta inhibition could prevent blood-retinal barrier (BRB) breakdown in diabetic retinopathy. Basically, these authors showed that PKC δ activation is involved in the decrease of tight junctions, particularly within the zona occludens -1 and -2, which is followed by BRB breakdown in mice. However, it is

well known [64] that VEGF *per se* reduces occludin content, therefore the modulation of VEGF expression appears the key point beyond the PKC isoforms. In addition, we cannot exclude that VEGF itself may activate PKC, as shown for PKC α [65], leading to a vicious circle.

The present data point, for the first time, to the existence, in the retina, of a new molecular pathway implicated in the control of VEGF expression and highlight that this molecular cascade can be affected by diabetes. Overall, these results lay the foundation to disclose new potential pharmacological targets useful to counteract pathologies implicating VEGF overexpression, such as the development and progression of diabetic retinopathy, and offer novel clues to study other complications associated to VEGF dysfunctions.

Acknowledgments

This work was supported by a grant from MIUR, PRIN 2007.

References

[1] Wilkinson-Berka JL, Miller AG. Update on the treatment of diabetic retinopathy. ScientificWorldJournal. 2008 Feb 6;8:98-120.

[2] Porta M, Bandello F. Diabetic retinopathy. A clinical update. Diabetologia 2002;45:1617-1634.

[3] Ferrara N. Vascular endothelial growth factor. Arterioscler Thromb Vasc Biol. 2009;29:789-791.

[4] Adamis AP, Shima DT. The role of vascular endothelial growth factor in ocular health and disease. Retina. 2005;25:111-118.

[5] Shibuya M. Vascular endothelial growth factor-dependent and –indipendent regulation of angiogenesis. BMB Rep. 2008;41:278-286.

[5] Michaelson IC. Vascular morphogenesis in the retina of the cat. J Anat. 1948;82:167-174.

[6] Wirostko B, Wong TY, Simó R. Vascular endothelial growth factor and diabetic complications. Prog Retin Eye Res. 2008;27:608-621.

[7] Penn JS, Madan A, Caldwell RB, Bartoli M, Caldwell RW, Hartnett ME. Vascular endothelial growth factor in eye disease. Prog Retin Eye Res. 2008;27:331-371.

Page 16 of 30

[8] Ye X, Xu G, Chang Q, Fan J, Sun Z, Qin Y, Jiang AC. ERK1/2 signaling pathways involved in VEGF release in diabetic rat retina. Invest Ophthalmol Vis Sci. 2010 May 5. [Epub ahead of print]

[9] Yokota T, Utsunomiya K, Taniguchi K, Gojo A, Kurata H, Tajima N. Involvement of the Rho/Rho kinase signaling pathway in platelet-derived growth factor BB-induced vascular endothelial growth factor expression in diabetic rat retina. Jpn J Ophthalmol. 2007;51:424-30.

[10] Clarke M, Dodson PM. PKC inhibition and diabetic microvascular complications. Best Pract Res Clin Endocrinol Metab. 2007;21:573-586.

[11] Pascale A, Amadio M, Govoni S, Battaini F. The aging brain, a key target for the future: the protein kinase C involvement. Pharmacol Res. 2007;55:560-569.

[12] Amadio M, Govoni S, Alkon DL, Pascale A. Emerging targets for the pharmacology of learning and memory. Pharmacol Res. 2004;50:111-122.

[13] Koya D, King GL. Protein kinase C activation and the development of diabetic complications. Diabetes. 1998;47:859-866.

[14] Idris I, Gray S, Donnelly R. Protein kinase C activation: isozyme-specific effects on metabolism and cardiovascular complications in diabetes. Diabetologia 2001;44:659-673.

[15] Aiello LP. The potential role of PKC beta in diabetic retinopathy and macular edema. Surv Ophthalmol. 2002;47:S263-269.

[16] Das Evcimen N, King GL. The role of protein kinase C activation and the vascular complications of diabetes. Pharmacol Res. 2007;55:498-510.

[17] Kim YH, Kim YS, Park CH et al. Protein kinase C-delta mediates neuronal apoptosis in the retinas of diabetic rats via the Akt signaling pathway. Diabetes. 2008;57:2181-2190.

[18] Amadio M, Scapagnini G, Lupo G, Drago F, Govoni S, Pascale A. PKCbetaII/HuR/VEGF: A new molecular cascade in retinal pericytes for the regulation of VEGF gene expression. Pharmacol Res 2008;57:60-66.

[19] Pascale A, Amadio M, Quattrone A. Defining a neuron: neuronal ELAV proteins. Cell Mol Life Sci. 2008;65:128-140.

[20] Bolognani F, Perrone-Bizzozero NI. RNA-protein interactions and control of mRNA stability in neurons. J Neurosci Res. 2008;86:481-489.

[21] Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. J Biol Chem. 1996;271:2746-2753.

[22] Levy NS, Chung S, Furneaux H, Levy AP. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem. 1998;273:6417-6423.

[23] Tang K, Breen EC, Wagner PD. Hu protein R-mediated posttranscriptional regulation of VEGF expression in rat gastrocnemius muscle. Am J Physiol Heart Circ Physiol. 2002;283:H1497-1504.

[24] Lejbkowicz F, Goldberg-Cohen I, Levy AP. New horizons for VEGF. Is there a role for nuclear localization? Acta Histochem. 2005;106:405-411.

[25] Slosberg ED, Yao Y, Xing F, Ikui A, Jirousek MR, Weinstein IB. The protein kinase C betaspecific inhibitor LY379196 blocks TPA-induced monocytic differentiation of HL60 cells. Mol Carcinog. 2000;27:166-176.

[26] Pascale A, Fortino I, Govoni S, Trabucchi M, Wetsel WC, Battaini F. Differential isoformspecific regulation of calcium-independent protein kinase C in rat cerebral cortex. Neurosci Lett. 1996;214:99-102.

[27] Ron D, Jiang Z, Yao L, Vagts A, Diamond I, Gordon A. Coordinated movement of RACK1 with activated betaII PKC. J Biol Chem. 1999;274:27039-27046.

[28] Pascale A, Gusev PA, Amadio M, Dottorini T, Govoni S, Alkon DL, Quattrone A. Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. Proc Natl Acad Sci U S A. 2004;101:1217-1222.

[29] Pascale A, Amadio M, Scapagnini G et al. Neuronal ELAV proteins enhance mRNA stability by a PKCalpha-dependent pathway. Proc Natl Acad Sci USA. 2005;102:12065-12070.

[30] Patel R, Pariente JA, Martinez MA, Salido GM, Singh J. Effect of insulin on acetylcholineevoked amylase release and calcium mobilization in streptozotocin-induced diabetic rat pancreatic acinar cells. Ann N Y Acad Sci. 2006;1084:58-70.

[31] Ishii H, Jirousek MR, Koya D et al. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. Science. 1996;272:728-731.

[32] Liu Y, Su W, Thompson EA, Leitges M, Murray NR, Fields AP. Protein kinase Cbeta II regulates its own expression in rat intestinal epithelial cells and the colonic epithelium in vivo. J Biol Chem. 2004;279:45556-45563.

[33] Kusari J, Zhou SX, Padillo E, Clarke KG, Gil DW. Inhibition of vitreoretinal VEGF elevation and blood-retinal barrier breakdown in streptozotocin-induced diabetic rats by brimonidine. Invest Ophthalmol Vis Sci. 2010;51:1044-1051.

[34] Xu X, Zhu Q, Xia X, Zhang S, Gu Q, Luo D. Blood-retinal barrier breakdown induced by activation of protein kinase C via vascular endothelial growth factor in streptozotocin-induced diabetic rats. Curr Eye Res. 2004;28:251-256.

[35] Cohen MP, Hud E, Wu VY, Shearman CW. Amelioration of diabetes-associated abnormalities in the vitreous fluid by an inhibitor of albumin glycation. Invest Ophthalmol Vis Sci. 2008;49:5089-5093.

[36] DCCT Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med. 1993;329: 977-986.

[37] Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615-1625.

[38] Suzuma K, Takahara N, Suzuma I et al. Characterization of protein kinase C beta isoform's action on retinoblastoma protein phosphorylation, vascular endothelial growth factor-induced endothelial cell proliferation, and retinal neovascularization. Proc Natl Acad Sci USA. 2002;99:721-726.

[39] Ohshiro Y, Ma RC, Yasuda Y et al. Reduction of diabetes-induced oxidative stress, fibrotic cytokine expression, and renal dysfunction in protein kinase C beta-null mice. Diabetes. 2006;55:3112-3120.

[40] Kim YH, Kim YS, Kang SS et al. Expression of 14-3-3 zeta and interaction with protein kinase C in the rat retina in early diabetes. Diabetologia 2005;48:1411-1415.

[41] Xia Z, Kuo KH, Nagareddy PR et al. N-acetylcysteine attenuates PKCbeta2 overexpression and myocardial hypertrophy in streptozotocin-induced diabetic rats. Cardiovasc Res. 2007;73:770-782.

[42] Langham RG, Kelly DJ, Gow RM et al. Increased renal gene transcription of protein kinase Cbeta in human diabetic nephropathy: relationship to long-term glycaemic control. Diabetologia. 2008;51:668-674.

[43] Joussen AM, Poulaki V, Mitsiades N, Kirchhof B, Koizumi K, Döhmen S, and Anthony P. Adamis AP. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNFα suppression. FASEB J. 2002;16:438-40.

[44] Aiello LP, Bursell SE, Clermont A et al. Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C in vivo and suppressed by an orally effective betaisoform-selective inhibitor. Diabetes. 1997;46:1473-1480.

[45] Budhiraja S, Singh J. Protein kinase C beta inhibitors: a new therapeutic target for diabetic nephropathy and vascular complications. Fundam Clin Pharmacol. 2008;22:231-240.

[46] Shibuya M. Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis. BMB Rep. 2008;41:278-286.

[47] Simó R, Carrasco E, García-Ramírez M, Hernández C. Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. Curr Diabetes Rev. 2006;2:71-98.

[48] Barreau C, Paillard L, Osborne HB. AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res. 2006;33:7138-7150.

[49] Short S, Tian D, Short ML, Jungmann RA. Structural determinants for post-transcriptional stabilization of lactate dehydrogenase A mRNA by the protein kinase C signal pathway. J Biol Chem. 2000;275:12963-12969.

[50] Rossi D, Amadio M, Carnevale Baraglia A et al. Discovery of Small Peptides Derived from Embryonic Lethal Abnormal Vision Proteins Structure Showing RNA-Stabilizing Properties. J Med Chem 2009 Jul 31 [Epub ahead of print].

[51] Wells DG. RNA-binding proteins: a lesson in repression. J Neurosci. 2006;26:7135-7138.

[52] Keene JD. Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. Proc Natl Acad Sci USA. 1999;96:5-7.

[53] Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G. Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimental background diabetic retinopathy of the rat. Diabetes. 1998;47:401-406.

[54] Wang K, Wang Y, Gao L, Li X, Li M, Guo J. Dexamethasone inhibits leukocyte accumulation and vascular permeability in retina of streptozotocin-induced diabetic rats via reducing vascular endothelial growth factor and intercellular adhesion molecule-1 expression. Biol Pharm Bull. 2008;31:1541-1546.

[55] Gerhardinger C, Costa MB, Coulombe MC, Toth I, Hoehn T, Grosu P. Expression of acutephase response proteins in retinal Mu[°]ller cells in diabetes. Invest Ophthalmol Vis Sci. 2005;46:349 -357.

[56] Brucklacher RM, Patel KM, VanGuilder HD, et al. Whole genome assessment of the retinal response to diabetes reveals a progressive neurovascular inflammatory response. BMC Med Genomics. 200813;1:26.

[57] Chung HK, Choi SM, Ahn BO, Kwak HH, Kim JH, Kim WB. Efficacy of troxerutin on streptozotocin-induced rat model in the early stage of diabetic retinopathy. Arzneimittelforschung. 2005; 55:573-580.

[58] Amadio M, Scapagnini G, Laforenza U et al. Post-transcriptional regulation of HSP70 expression following oxidative stress in SH-SY5Y cells: the potential involvement of the RNAbinding protein HuR. Curr Pharm Des. 2008;14:2651-2658.

[59] Danilin S, Sourbier C, Thomas L, Lindner V, Rothhut S, Dormoy V, Helwig JJ, Jacqmin D, Lang H, Massfelder T. Role of the RNA-binding protein HuR in human renal cell carcinoma. Carcinogenesis. 2010;31:1018-1026.

[60] McMullen MR, Cocuzzi E, Hatzoglou M, Nagy LE. Chronic ethanol exposure increases the binding of HuR to the TNFalpha 3'-untranslated region in macrophages. J Biol Chem. 2003;278:38333-38341.

[61] Gantt KR, Cherry J, Richardson M, Karschner V, Atasoy U, Pekala PH. The regulation of glucose transporter (GLUT1) expression by the RNA binding protein HuR. J Cell Biochem. 2006; 99:565-574.

[62] Ayalasomayajula SP, Ashton P, Kompella UB. Fluocinolone inhibits VEGF expression via glucocorticoid receptor in human retinal pigment epithelial (ARPE-19) cells and TNF-alphainduced angiogenesis in chick chorioallantoic membrane (CAM). J Ocul Pharmacol Ther. 2009;25:97-103.

[63] Kim JH, Kim JH; Jun HO, Yu YS, Kim KW. Inhibition of protein kinase C delta attenuates blood-retinal barrier breakdown in diabetic retinopathy. Am J Pathol. 2010;176:1517-1524.

[64] Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW. Vascular permeability in experimental diabetes associated with reduced endothelial occludin content. Diabetes 1998;47:1953-1959.

[65] Podar K, Tai YT, Lin BK, Narsimhan RP, Sattler M, Kijima T, Salgia R, Gupta D, Chauhan D, Anderson KC. Vascular endothelial growth factor-induced migration of multiple myeloma cells is associated with beta 1 integrin- and phosphatidylinositol 3-kinase-dependent PKC alpha activation. J Biol Chem. 2002; 277:7875-7881.

Table 1. *Effects of STZ-induced diabetes on body weight and blood glucose levels in different groups after ten days.* Control group are normal rats injected with the vehicle used to dissolve STZ. LY379196, a selective inhibitor of PKC β , was given at 1mg/kg (i.p.) per day. Diabetes was induced by 60 mg/kg (i.v.) injection of STZ.

Groups	Body weight (g)	Nonfasting blood glucose (mg/dl)
Control (non-diabetic)	240 ± 21	99 ± 14
Diabetic	185 ± 16	408 ± 23*
Diabetic + LY 379196	190 ± 18	399 ± 40

Data are expressed as mean ± SD. *p<0.0001 vs control; Dunnett Multiple Comparisons test, n=10.

LEGENDS TO FIGURES

Fig. 1. *PKCβI and PKCβII increase in the retina from diabetic rats*. Representative western blottings of PKCβI (**panel A**, upper) and PKCβII (**panel B**, upper) and the respective α -tubulin in the retina from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (INHIB) rats. Mean grey level ratios (mean \pm S.E.M.) of PKCβI/ α -tubulin (**panel A**, lower) and PKCβII/ α -tubulin (**panel B**, lower) immunoreactivities measured by western blotting in the same samples. *p<0.05, **p<0.01 *vs.* CON; Dunnett Multiple Comparisons test, n=6-9.

Fig. 2. Upregulation and PKC-mediated phosphorylation of HuR in the retina from diabetic rats. (Panel A) Representative western blottings of HuR (upper) and the respective α -tubulin in the retina from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (INHIB) rats. Mean grey level ratios (mean \pm S.E.M.) of HuR/ α -tubulin (lower) immunoreactivities measured by western blotting in the same samples. **p<0.01 vs. sham; Dunnett Multiple Comparisons test, n=6-9. (Panel B, upper) Representative western blotting of phosphorylated serine (pSer) residues present in immunoprecipitated HuR protein in the retina from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (INHIB) rats. An irrelevant isotype-matched IgG (IRR) has been used as negative control. The samples were normalized according to α -tubulin values measured on the input signals. The Positive Control (PC) represents the band recognized by the anti-HuR antibody in a total retinal homogenate. (Panel B, lower) Mean grey level ratios (mean \pm S.E.M.) of p-Ser/ α -tubulin immunoreactivities measured by western blotting in the same samples. **p<0.01 vs. CON; Dunnett Multiple Comparisons test, n=4.

Fig. 3. *Total VEGF mRNA levels do not change following diabetes*. Representative Real Time-PCR melting curves (**Panel A**) and the corresponding amplification plots (**Panel B**) of VEGF in the retina from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (INHIB) rats. All the samples yield only one peak resulting from the specific amplification product corresponding to VEGF. For the blank sample, the template was replaced with PCR-grade water.) *HuR protein binds to VEGF mRNA in the rat retina*. Representative Real Time-PCR amplification plots (**Panel C**) of VEGF in the retinal mRNPs from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (INHIB) rats following immunoprecipitation experiments using the anti-HuR antibody (+HuR) or an irrelevant antibody (+IRR) with the same isotype of HuR as a negative control. (**Panel D**) Representative experiment showing the control samples (+HuR or +IRR) subjected to RT-PCR run in an agarose gel. A cDNA obtained from a total mRNA extract was utilized as a positive control.

Fig. 4. VEGF levels increase in the retina from diabetic rats. (Panel A) Representative western blottings of VEGF and the respective α -tubulin (upper) in the retina from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (INHIB) rats. Mean grey level ratios (mean \pm S.E.M.) of VEGF/ α -tubulin (lower) immunoreactivities measured by western

blotting in the same samples. *p<0.05 vs. CON; Dunnett Multiple Comparisons test, n=6. (**Panel B**) Retinal levels of VEGF were measured via ELISA ten days after streptozotocin (STZ) injection with or without LY379196 (INHIB) treatment. ***P < 0.001 vs. CON; Dunnett Multiple Comparisons test, n=5-8.

Fig. 5. The PKC β /HuR/VEGF pathway. Flow chart depicting the major events and possible additional mechanisms involved in this molecular cascade. For more details see the text.

م م م



Figure 1

K

A



Figure 2



Figure 3

100 bp



Figure 4





