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The PKC β /HuR/VEGF pathway in diabetic retinopathy

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ABSTRACT

1
2 We investigated whether the diabetes-related PKC β activation affects VEGF expression through the
3 mRNA-stabilizing human *Embryonic Lethal Abnormal Vision* (ELAV) protein, HuR, in the retina
4 of streptozotocin (STZ)-induced diabetic rats. Diabetes was induced in rats by STZ injection.
5 Retinal tissues were processed to detect PKC β I, PKC β II, VEGF and HuR contents, as well as HuR
6 phosphorylation. Immunoprecipitation coupled to RT-PCR was employed to evaluate HuR binding
7 to VEGF mRNA in RiboNucleoProteic (RNP) complexes. Statistical analysis was performed by
8 ANOVA followed by an appropriate *post hoc* comparison test. Following experimental diabetes
9 PKC β I and PKC β II levels were increased compared to sham; there was also a PKC-mediated
10 phosphorylation/activation of HuR. These effects were blunted by the *in vivo* co-administration of a
11 selective PKC β inhibitor. A specific binding between the HuR protein and the VEGF mRNA was
12 also detected. The PKC β /HuR activation was accompanied by enhanced VEGF protein expression
13 that was, again, blunted by the PKC β inhibitor. These findings first demonstrate the activation, in
14 the retina, of the PKC β /HuR/VEGF pathway following experimental diabetes and disclose a new
15 potential pharmacological target to counteract pathologies implicating VEGF deregulation, such as
16 diabetic retinopathy.
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23 **Keywords:** Diabetes; Embryonic Lethal Abnormal Vision protein; PKC; VEGF; Retina.
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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-insulin-dependent 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, and it 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

1 the basement membrane, hyper-permeability and formation of microaneurysms. These functional
2 alterations are followed by microvascular occlusions leading to a progressive retinal ischemia that
3 induces the release of the Vascular Endothelial Growth Factor (VEGF), also known as vascular
4 permeability factor. VEGF is a potent mitogen for endothelial cells that triggers proliferation,
5 migration and tube formation leading to growth of new blood vessels that, however, in the diabetic
6 retina are fragile and may break. VEGF (also called VEGF-A) is the major regulator of
7 physiological and pathological angiogenesis [3], and belongs to a family that includes placental
8 growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E [4,5]. In 1948 Michaelson first
9 suggested the implication of a generic angiogenic factor in the neovascularization process occurring
10 in retinopathy [5]; nowadays several studies stress that a key role in the development of diabetic
11 retinopathy is indeed played by VEGF [6,7]. The expression of VEGF in diabetic retina can be
12 regulated by different pathways, , such as Rho/Rho Kinase, ERK1/2, and Protein Kinase C (PKC)
13 [8-10]. PKC is a family of at least 10 serine-threonine kinases ubiquitously expressed and able to
14 participate in multiple cellular functions [11,12]. Among the different PKC isoenzymes, the beta
15 (PKC β) one seems to be preferentially activated in the retina, thus possibly contributing to the early
16 stages of retinopathy [13,16], although other PKC isoenzymes are also activated such as alpha and
17 delta [16,17]. To date no findings are available on how PKC may exert its modulation on VEGF
18 expression. However, a clue comes from our previous *in vitro* data suggesting the existence, in
19 retinal pericytes, of a new molecular cascade involving PKC β , the mRNA-Binding Protein (RBP)
20 HuR and VEGF [18]. In that paper, we showed the presence of a new molecular cascade operating
21 in the cytoskeleton of retinal bovine pericytes controlling VEGF expression, although it should be
22 stressed that the study [16] was carried out in a milieu quite different from an *in vivo* system. HuR
23 is the ubiquitously expressed member of the ELAV (Embryonic Lethal Abnormal Vision) family,
24 highly conserved RBPs which act post-transcriptionally as positive regulators of gene expression
25 [19,20]. The relevance of post-transcriptional events in the regulation of VEGF production is

1 emphasized by the observation that while in normal conditions VEGF mRNA is extremely labile,
2 its half-life can increase during hypoxia, such as following retinal capillaries occlusion, by 2-3 fold
3
4 due to the stabilizing effect of HuR [21,22]. Moreover, Tang et al. [23] demonstrated that acute
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6 ischemia induces a rapid binding of HuR to the VEGF mRNA leading to increased levels of the
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8 corresponding VEGF protein. HuR seems to sterically protect VEGF mRNA from ribonucleases
9
10 [24], by masking specific instability signals within the VEGF mRNA sequence itself, thus, on the
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12 one hand, reducing its decay rate and, on the other, enhancing its translation. The purpose of the
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14 present work was to investigate whether the PKC β /HuR/VEGF pathway, previously described *in*
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16 *vitro*, is operant also *in vivo* in the retina and altered by diabetes.
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23 **2. Materials and methods**

24 *2.1. Animals and reagents*

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26 Male Sprague-Dawley rats weighing approximately 200 g were obtained from Charles River
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28 (Calco, Italy). All the animals were treated according to the ARVO (Association for Research in
29
30 Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.
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32 The animals were fed on standard laboratory food and were allowed free access to water in an air-
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34 conditioned room with a 12-h light/12-h dark cycle. Final group sizes for all measurements were $n=$
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36 6-9 except as noted. Streptozotocin (STZ) and phenyl-methyl sulfonyl fluoride were purchased
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38 from Sigma-Aldrich (Milan, Italy). LY379196, a bisindolylmaleimide derivative of staurosporine
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40 that shows high selectivity for the β isoforms of PKC [25], was a kind gift of Eli-Lilly, and was
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42 used as tool in all experiments. All the other reagents were obtained from Sigma-Aldrich (Milan,
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44 Italy) unless otherwise stated.
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52 *2.2. Induction of diabetes*

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

32 2.3. Preparation of the samples and western blotting

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Rat retinæ 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-PKC β I mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted at 1:500, the anti-PKC β II 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-

1 α -tubulin rat monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) at 1:1000.

2 The nitrocellulose membrane signals were detected by chemiluminescence. Experiments were
3 performed at least three times for each cell preparation; the same membranes were re-probed with
4 α -tubulin antibody and used to normalize the data. Statistical analysis of western blot data was
5 performed on the densitometric values obtained with the NIH image software 1.61 (downloadable at
6 <http://rsb.info.nih.gov/nih-image>).
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14 2.4. Preparation of mRNP (RiboNucleoProteic complexes)

15 The retinal tissues were homogenized in ice-cold polysome lysis buffer [containing 100
16 mM KCl, 5 mM MgCl₂, 10 mM Hepes (pH 7), 0.5% Nonidet P-40 and 1 mM DTT (Dithiothreitol)]
17 supplemented with RNase and protease inhibitors, mixed by pipetting several times, placed on ice
18 for 10 min, frozen and stored at -80° C until use. The homogenates were thawed and centrifuged in
19 a tabletop microcentrifuge at 14,000 x g for 10 min at 4° C. The supernatant was removed and
20 centrifuged again in the same manner. The final supernatant, representing a fraction particularly
21 enriched in mRNAs and proteins (called mRNP), was stored at -20° C until use for
22 immunoprecipitation experiments.
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38 2.5. Immunoprecipitation

39 Samples (total lysates or mRNP) were processed according to a previously published
40 protocol with minor modifications [27]. Briefly, immunoprecipitation was performed at room
41 temperature for 2 h using 1 μ g of anti-HuR antibody (Santa Cruz Biotechnology, Santa Cruz, CA,
42 USA) per 50 μ g of proteins diluted with an equal volume of 2x Immunoprecipitation Buffer [2%
43 Triton X-100, 300mM NaCl, 20mM Tris-HCl (pH 7.4), 2mM EDTA, 2mM EGTA, protease
44 inhibitor cocktail and an RNAase inhibitor] in presence of 50 μ l of protein A/G plus agarose (Santa
45 Cruz Biotechnology, Santa Cruz, CA USA) previously blocked with 5% BSA in PBS. The samples
46 were finally subjected to either western blotting or RNA extraction. The western blottings on the
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1 immunoprecipitated proteins were performed using the mouse antibodies anti-phosphothreonine
2 diluted at 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA USA) and anti-phosphoserine diluted
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4 at 1:500 (Sigma-Aldrich, Milan, Italy). The negative control was obtained under identical
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6 conditions, but in the presence of an irrelevant antibody (IRR) with the same isotype of the specific
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8 immunoprecipitating antibody. Small aliquots of the immunoprecipitation mixes were collected
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10 from each sample and used as “input signals” (I.S.) to normalize the western blotting or the Real
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12 Time-PCR (RT-PCR) data. The Positive Control (PC) was represented by a total retinal
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14 homogenate obtained from additional control rats, homogenized in Buffer A, and processed as
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16 described in the section 2.3.
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22 2.6. *Quantitative Real Time-PCR*

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24 RNA was extracted from total homogenates and immunoprecipitated pellets using RNeasy
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26 Micro Kit (Qiagen, Milan, Italy). The reverse transcription was performed following standard
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28 procedures. PCR amplifications were carried out using the Lightcycler instrument (Roche
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30 Molecular Biochemicals, Mannheim, Germany) in the presence of FastStart SYBR Green master
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32 mix (Roche Molecular Biochemicals, Mannheim, Germany) and with the specific primers for
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34 VEGF: Forward: 5'-TCACCAAAGCCAGCACATAG-3'; Reverse: 5'-
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36 AACAAAGGCTCACAGTGATTTTCT-3'. The GAPDH (Glyceraldehyde 3-Phosphate
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38 Dehydrogenase) mRNA, whose primers are: 5'-CAGCAAGGATACTGAGAGCAAG-3'
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40 (upstream), and 5'-GGATGGAATTGTGAGGGAGA-3' (downstream), was chosen as reference
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42 [28] on which VEGF values were normalized and to check the specificity of the HuR binding to
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44 VEGF. The quantification (expressed in Cycle threshold, Ct) of VEGF mRNA in the total
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46 homogenates and immunoprecipitated pellets was performed as previously published [29].
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48 Moreover, as concerns the immunoprecipitated pellets the amplified products were also run in an
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50 agarose gel.
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2.7. ELISA assay for VEGF

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2 Retinal tissues were homogenized in 100 μ l of solution consisting of 20 mM imidazole
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4 hydrochloride, 100 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1% Triton, 10 mM NaF, 1 mM sodium
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6 molybdate, and 1 mM EDTA. The solution was supplemented with a cocktail of protease
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8 inhibitors (Complete, Roche, Basel, Switzerland) before use. The samples were cleared *via*
9
10 centrifugation for 10 min at 10,000 \times g and assessed for protein concentration with the
11
12 bicinchoninic acid (BCA) assay (Mini BCA kit; Pierce Scientific, Rockford, IL, USA). The VEGF
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14 protein levels were estimated with the respective ELISA kit (R&D Systems Inc., Minneapolis, MN,
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16 USA), according to the manufacturer's instructions. All the measurements were performed in
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18 duplicate. The tissue sample concentration was calculated from a standard curve and corrected for
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20 protein concentration.
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2.8. Data analysis

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28 For statistical analysis the GraphPad InStat statistical package (version 3.05 GraphPad
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30 software, San Diego, CA, USA) was used. The data were analyzed by analysis of variance
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32 (ANOVA) followed, when significant, by an appropriate *post hoc* comparison test. Differences
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34 were considered statistically significant when p values \leq 0.05.
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3. Results

3.1. Glycemia and Body Weight

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43 Induction of diabetes with STZ is associated with hyperglycemia and weight loss. After ten
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45 days from STZ administration, blood glucose values in diabetic rats were significantly higher than
46
47 the corresponding values in non-diabetic animals (408 ± 23 and 99 ± 14 mg/dl respectively; see
48
49 Table 1). A decrease in body weight of diabetic rats was observed, as also reported by others [30].
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51 The PKC β selective inhibitor LY379196 did not interfere with glycemia and body weight (Table
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53 1), this finding is in agreement with literature data showing that the repeated administration of a
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2 specific PKC β inhibitor, closely related to LY379196, does not induce changes in body weight or
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5 glucose levels in rats [31].

6 3.2. Upregulation of PKC β and phosphorylation of HuR in the retina of diabetic rats

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8 Considering that PKC signal transduction is enhanced in the hyperglycemic state [16], and
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10 that PKC β isoforms are especially implicated in diabetic retinopathy, we first evaluated the protein
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12 content of both PKC β I and β II in retinal tissues from STZ-induced diabetic rats. As shown in Fig. 1,
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14 both PKC β I and β II isotypes increased following streptozotocin with respect to sham animals
15
16 (+160%, Fig. 1A; +113%, Fig. 1B). This up-regulation is possibly related to the activated PKC β
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18 that is able to stimulate its own transcription leading to an enhanced production of the
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20 corresponding protein [32]. Indeed, this effect was counteracted by the *in vivo* co-administration of
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22 the selective PKC β inhibitor LY379196 (Fig. 1A and 1B). We previously found *in vitro* evidence
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24 that PKC β and HuR belong to the same molecular cascade [18]; it was then of great interest to
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26 verify the existence of this pathway *in vivo* exploring HuR protein content and its PKC-dependent
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28 phosphorylation (as an indirect index of kinase activity). As reported in Fig. 2, HuR protein levels
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30 increased in STZ-treated rats (+62% vs. control, Fig. 2A) and, more interestingly, this was
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32 accompanied by a higher phosphorylation in serine residues (+209% vs. control, Fig. 2B). This last
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34 finding is in line with the high number of serine residues within the aminoacidic sequence of HuR
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36 and with our previous results in retinal pericytes [18]. The effects on the HuR protein were
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38 specifically related to PKC β since they were blunted by the selective inhibitor LY379196 (Fig. 2A
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40 and 2B).
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50 3.3. Effect of the PKC β /HuR cascade activation on VEGF

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52 We previously demonstrated that in retinal bovine pericytes PKC β activation is able,
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54 through the RNA-binding protein HuR, to increase VEGF protein levels [18]. Given the triggering
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56 of the PKC β /HuR cascade in retinal tissues from diabetic rats, we investigated its consequences on
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1 VEGF expression. We first evaluated the content of VEGF mRNA in retinal tissues from control
2 and STZ-induced diabetic rats to explore whether diabetes influences VEGF transcription. The
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4 results indicate that total VEGF mRNA levels do not change following diabetes, moreover they are
5
6 not even affected by the concomitant treatment with LY379196 (*mean of Ct values*: CON= $28.8 \pm$
7
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
9
10 suggest that in our model VEGF expression is not modified at the transcriptional level and
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12 prompted us to investigate whether this gene undergoes a post-transcriptional regulation. We thus
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14 initially examined the existence of a specific binding between the HuR protein and VEGF mRNA in
15
16 the retina. For this we isolated the mRNPs, performed immunoprecipitation with the anti-HuR
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18 antibody and finally detected the VEGF mRNA by RT-PCR. Immunoprecipitation with an
19
20 irrelevant antibody with the same isotype of HuR antibody was used as a negative control. The
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22 amplification curves indicate that the amount of VEGF transcript is much higher in the samples
23
24 immunoprecipitated with the anti-HuR antibody (Fig. 3C). These results are supported by the
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26 observation that when the amplified products from RT-PCR were run in an agarose gel, VEGF
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28 mRNA is only detectable in the anti-HuR immunoprecipitated samples (Fig. 3D), thus indicating
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30 the specificity of the binding between the HuR protein and VEGF mRNA. Additionally, as
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32 expected, in these same samples the GAPDH mRNA was almost undetectable (not shown). The
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34 RT-PCR analysis on the anti-HuR immunoprecipitated samples from control and treated (STZ and
35
36 STZ+LY379196) animals indicates that the amount of VEGF mRNA bound to HuR protein is
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38 similar in the all retinal tissues, although a non significant slight increase was observed in STZ-
39
40 induced diabetic rats in comparison to sham (STZ vs. CON +12%; STZ+LY379196 vs. CON +3%;
41
42 n=6). We finally evaluated the effects of diabetes on VEGF translation by western blotting. The
43
44 results point out that VEGF protein content is significantly increased in STZ-induced diabetic rats
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46 (+256% vs. control, Fig. 4A); again, the *in vivo* co-administration of the selective inhibitor
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48 LY379196 restores the VEGF protein levels to normal conditions (Fig. 4A).
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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

1 dramatic increase in the angiogenic response to retinal ischemia in transgenic mice overexpressing
2 PKC β and, on the other hand, by a significant decrease in retinal neovascularization in PKC β null
3 mice [38]. Moreover, besides the retina, it has been observed that the lack of PKC β can protect
4 against diabetic-induced dysfunctions and augment VEGF expression in other tissues affected by
5 this pathology [39]. However, in the retina and in other districts there is evidence indicating that
6 diabetes is associated not only with an increase of PKC activation, but also with augmented PKC
7 protein levels [16,40,41]. Our present data are in line with these findings, showing that in the retina,
8 as a consequence of diabetes, there is an increased amount of both PKC β I and PKC β II proteins. As
9 regards the underlying mechanism, it has been suggested [31] that the activated PKC β is able to
10 stimulate its own transcription leading to an increased expression of the corresponding protein (see
11 also Fig. 5). In line with this hypothesis, a recent paper reports an enhanced transcription of the
12 PKC β gene in kidney biopsies of diabetic patients compared to normal controls [42]. In agreement
13 with our results, Liu et al. [32] also showed that the up-regulation of PKC β protein levels is blunted
14 by a selective inhibitor of this kinase.

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

1 independent mechanisms [46,47]. On the other hand, VEGF mRNA bears a characteristic motif
2 found in its 3'-untranslated region, called Adenine uridine-Rich Element (ARE), which generally
3 governs the decay rates of specific mRNAs and represents the docking site for some RBPs [48]. It
4 has been shown that, in different cellular models, PKC activation induces the stabilization of ARE-
5 bearing mRNAs [19,49]. Specifically, our previous *in vitro* work in retinal bovine pericytes
6 describes a new molecular cascade involving PKC β , the RBP HuR, and VEGF [18] and supports
7 the key importance of post-transcriptional mechanisms in the modulation of VEGF expression [21-
8 23,50].

19 The post-transcriptional control is a "housekeeping" phenomenon that modifies the content
20 of a particular protein through a modulation of the half-life and/or the translation of the
21 correspondent mRNA. At this regard, RBPs play a critical role being able to affect the processing of
22 selected transcripts, their transport and localization in the cytoplasm, their stability and translation,
23 thus representing the most important *trans*-acting factors in the control of gene expression
24 [19,20,51]. The best characterized RBPs are the ELAV proteins that in the genomes of vertebrates
25 include neuron-specific members (HuB, HuC and HuD) and the ubiquitously expressed HuR [52].

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

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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 PKC β 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.

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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 α [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

1 well known [64] that VEGF *per se* reduces occludin content, therefore the modulation of VEGF
2 expression appears the key point beyond the PKC isoforms. In addition, we cannot exclude that
3 VEGF itself may activate PKC, as shown for PKC α [65], leading to a vicious circle.
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7 The present data point, for the first time, to the existence, in the retina, of a new molecular
8 pathway implicated in the control of VEGF expression and highlight that this molecular cascade can
9 be affected by diabetes. Overall, these results lay the foundation to disclose new potential
10 pharmacological targets useful to counteract pathologies implicating VEGF overexpression, such as
11 the development and progression of diabetic retinopathy, and offer novel clues to study other
12 complications associated to VEGF dysfunctions.
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23 **Acknowledgments**

24 This work was supported by a grant from MIUR, PRIN 2007.
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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 \pm 21	99 \pm 14
Diabetic	185 \pm 16	408 \pm 23*
Diabetic + LY 379196	190 \pm 18	399 \pm 40

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

LEGENDS TO FIGURES

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

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

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

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

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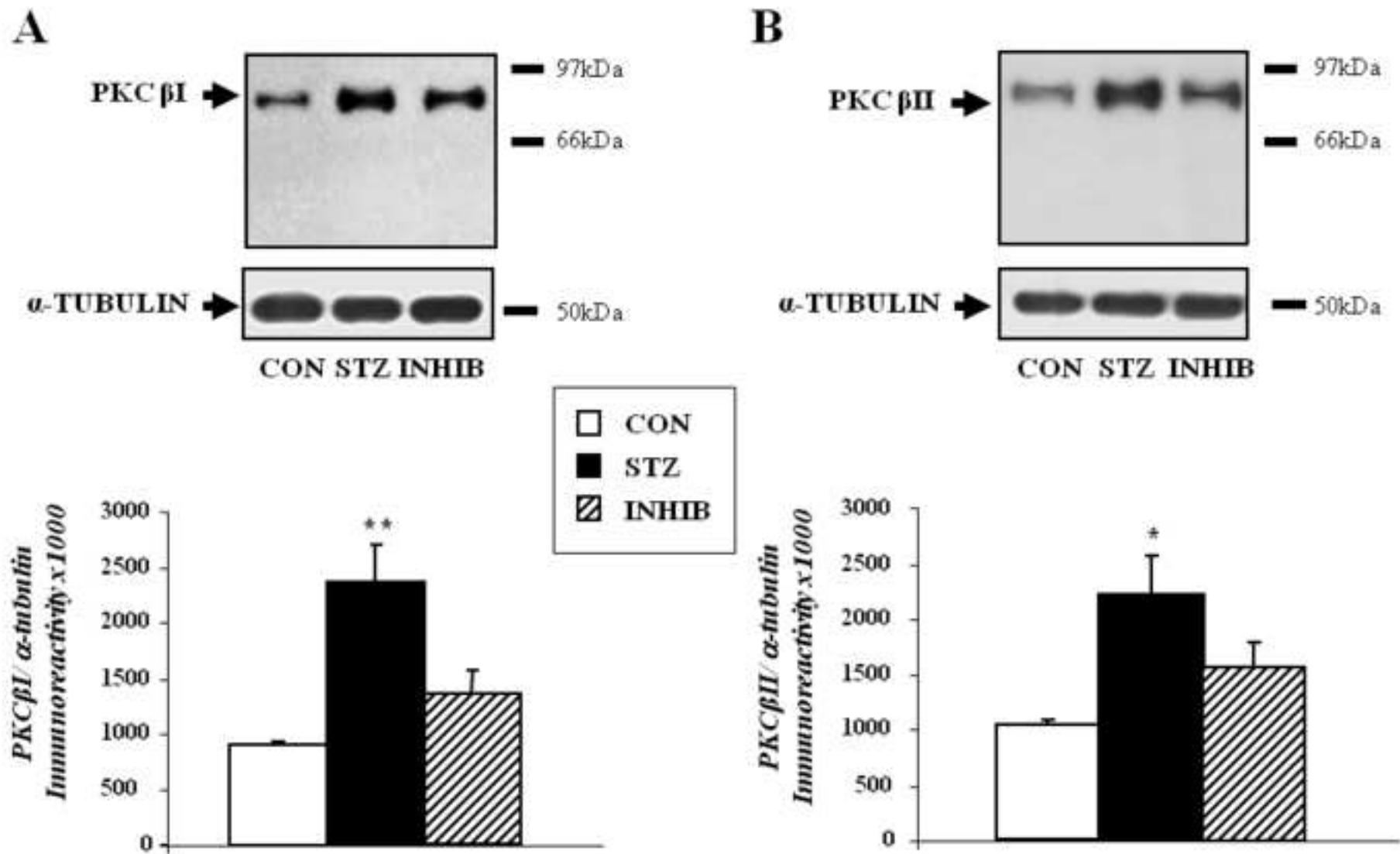


Figure 1

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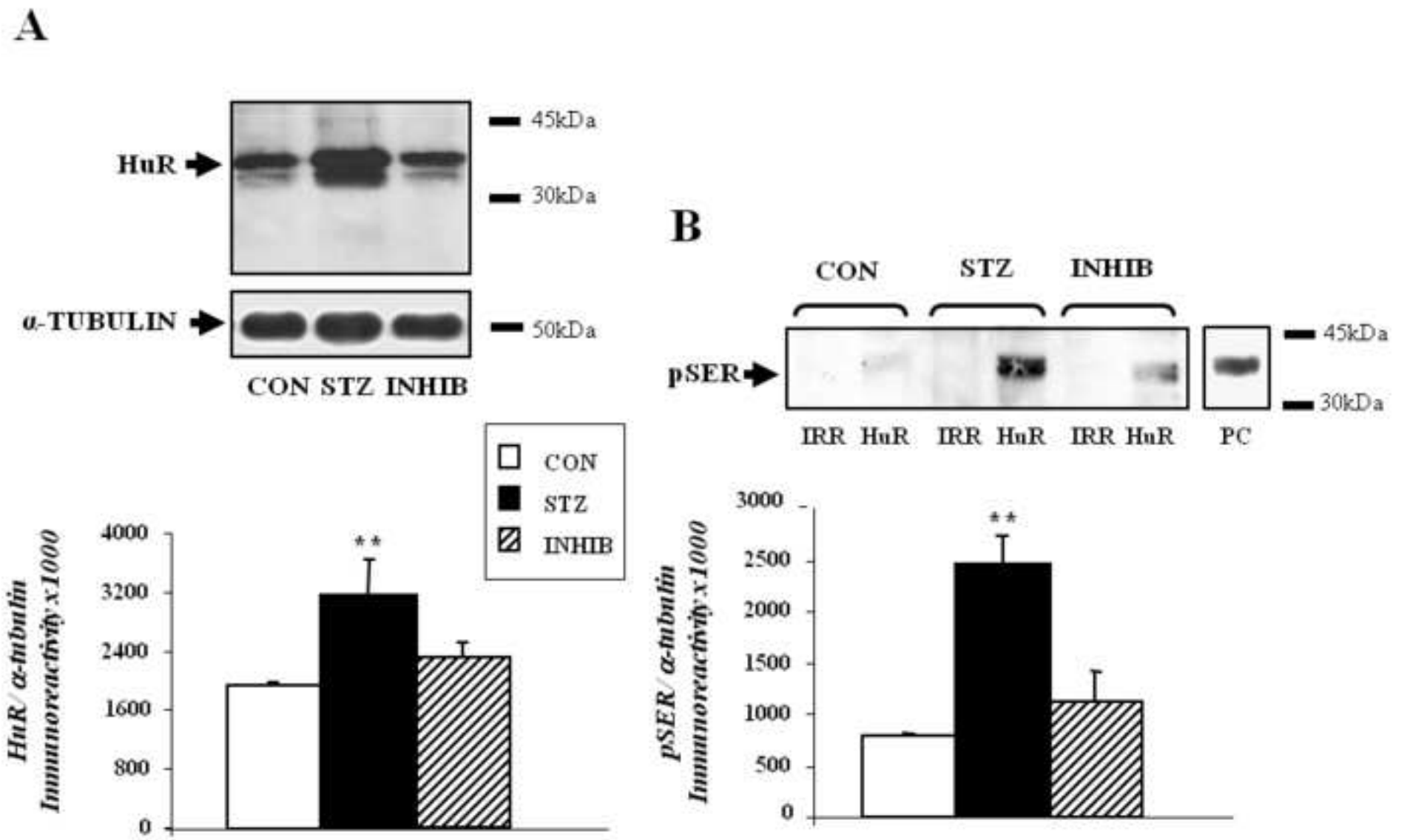


Figure 2

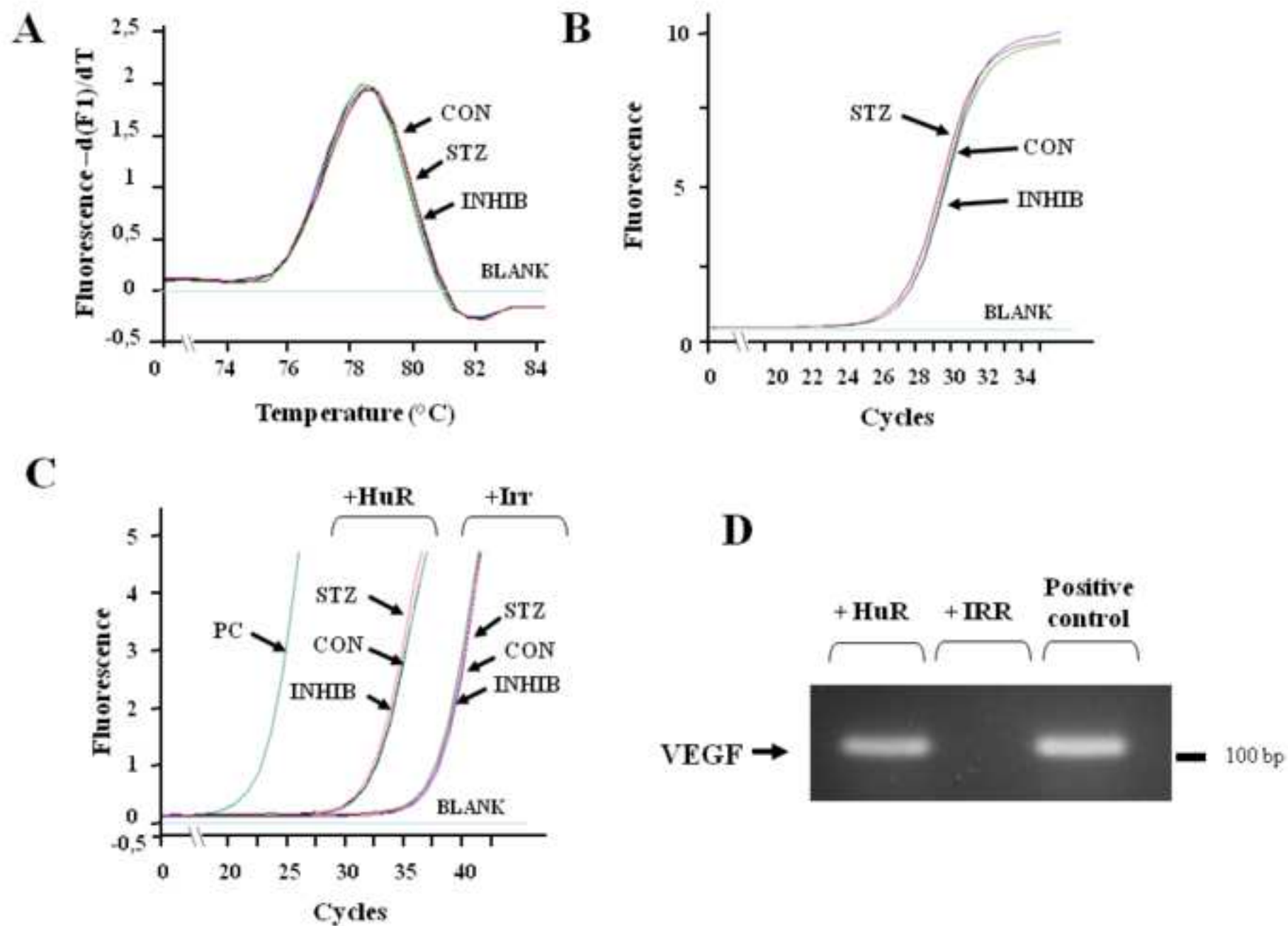


Figure 3

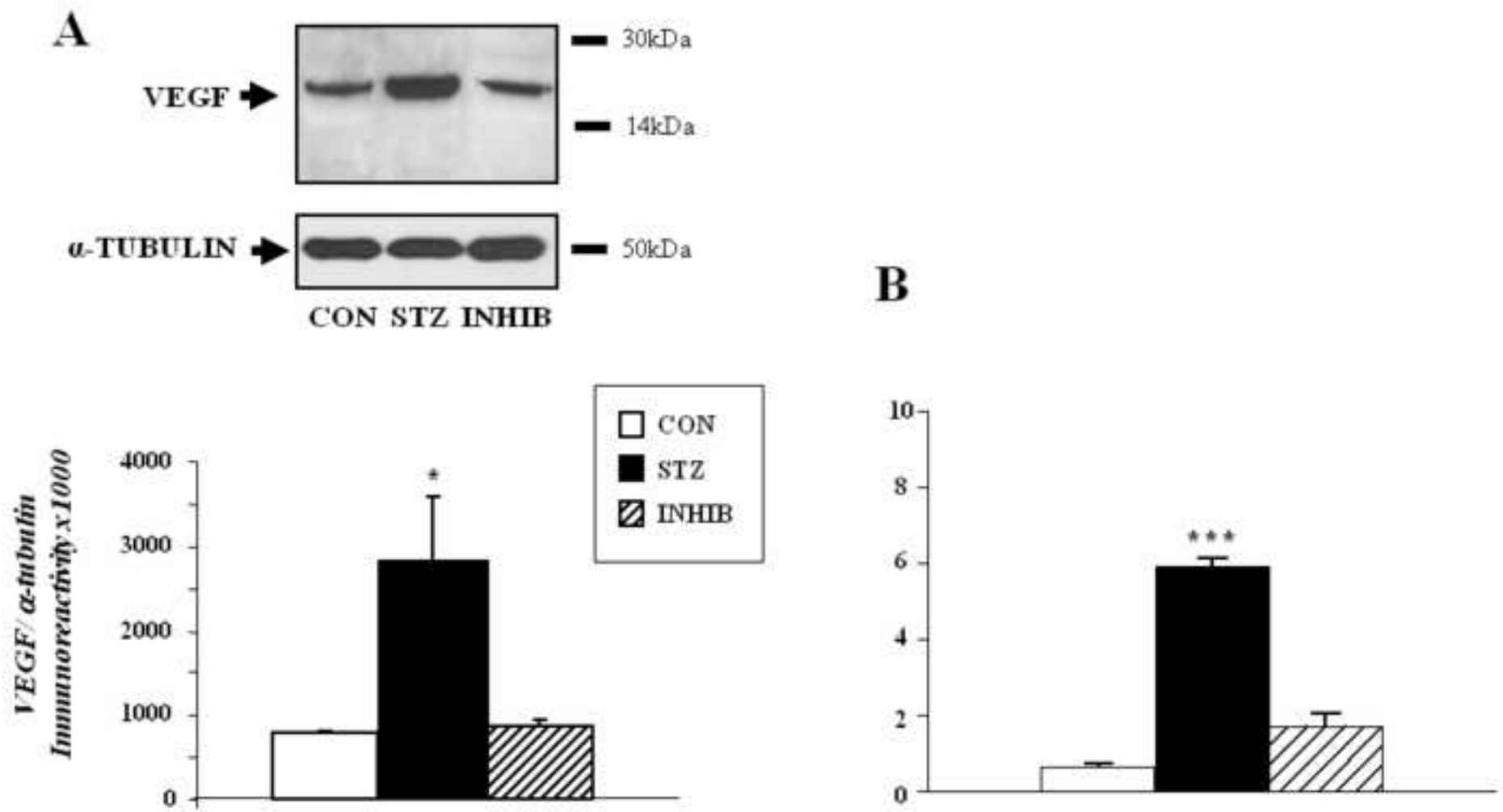


Figure 4

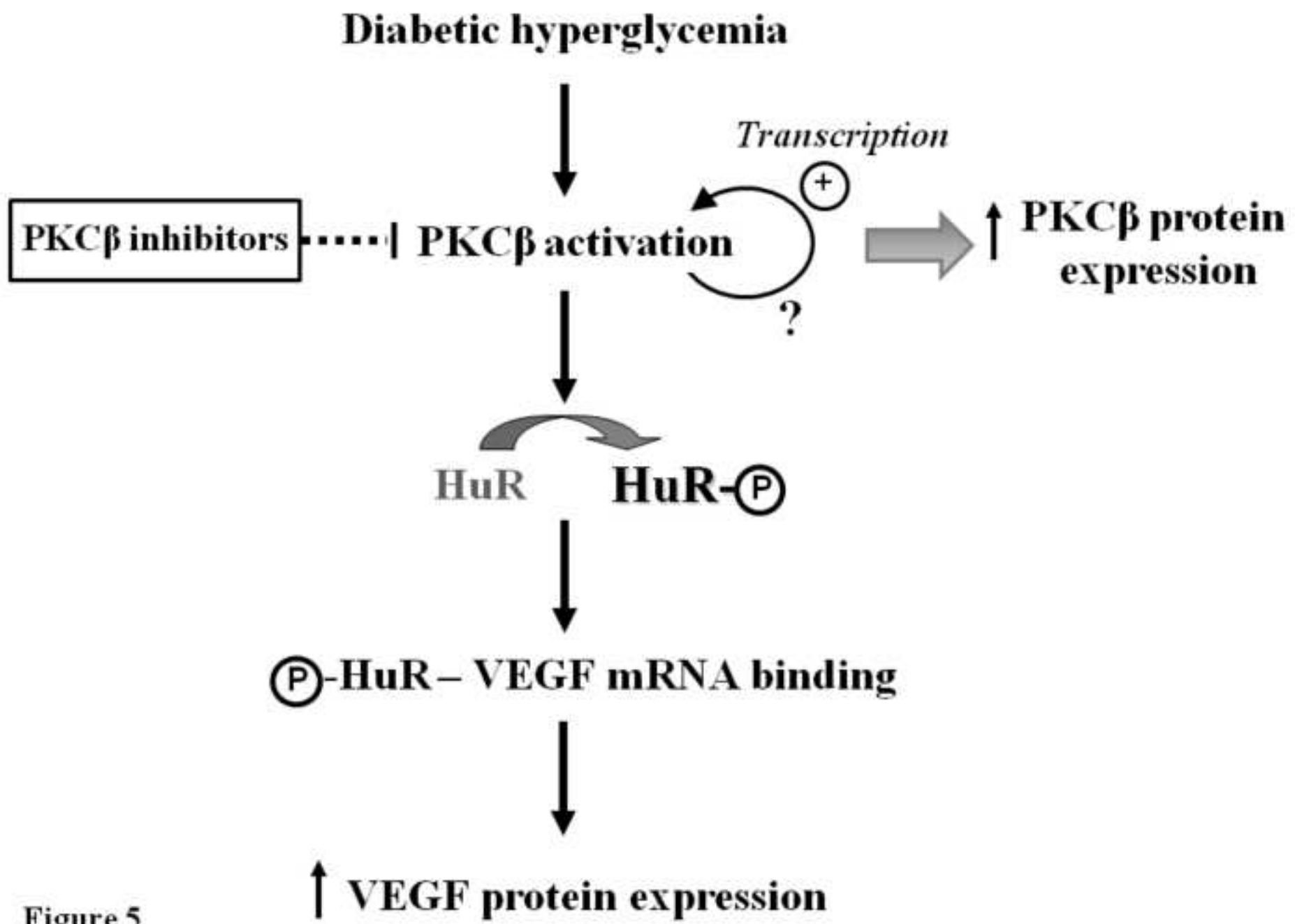


Figure 5

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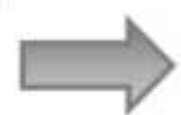
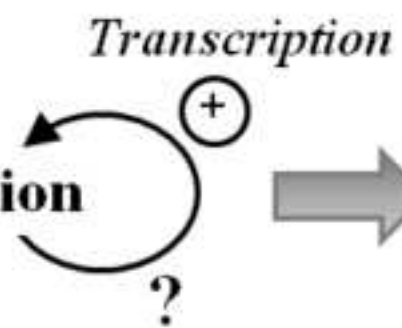
Diabetic hyperglycemia



PKCβ inhibitors



PKCβ activation



↑ PKCβ protein expression



Ⓟ-HuR – VEGF mRNA binding



↑ VEGF protein expression