HuR protein and diabetic retinopathy

Alessia Pascale

Dipartimento di Scienze del Farmaco, Sezione di Farmacologia, Università di Pavia.

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ELAV proteins

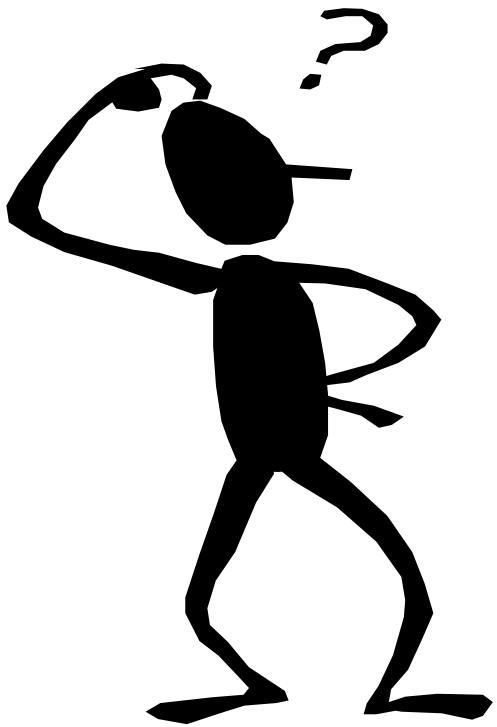
(*Embryonic Lethal Abnormal Vision*)

✓ **ELAV** or **Hu proteins** are a small family of **RNA binding proteins** whose genes represent the vertebrate homologues of the *elav* gene of Drosophila. The *elav* mutation produces in the fly embryonic lethality and abnormal development of neural tissue.

✓ In mammals four ELAV proteins have been identified:

HuB
HuC } **neuron-specific**
HuD

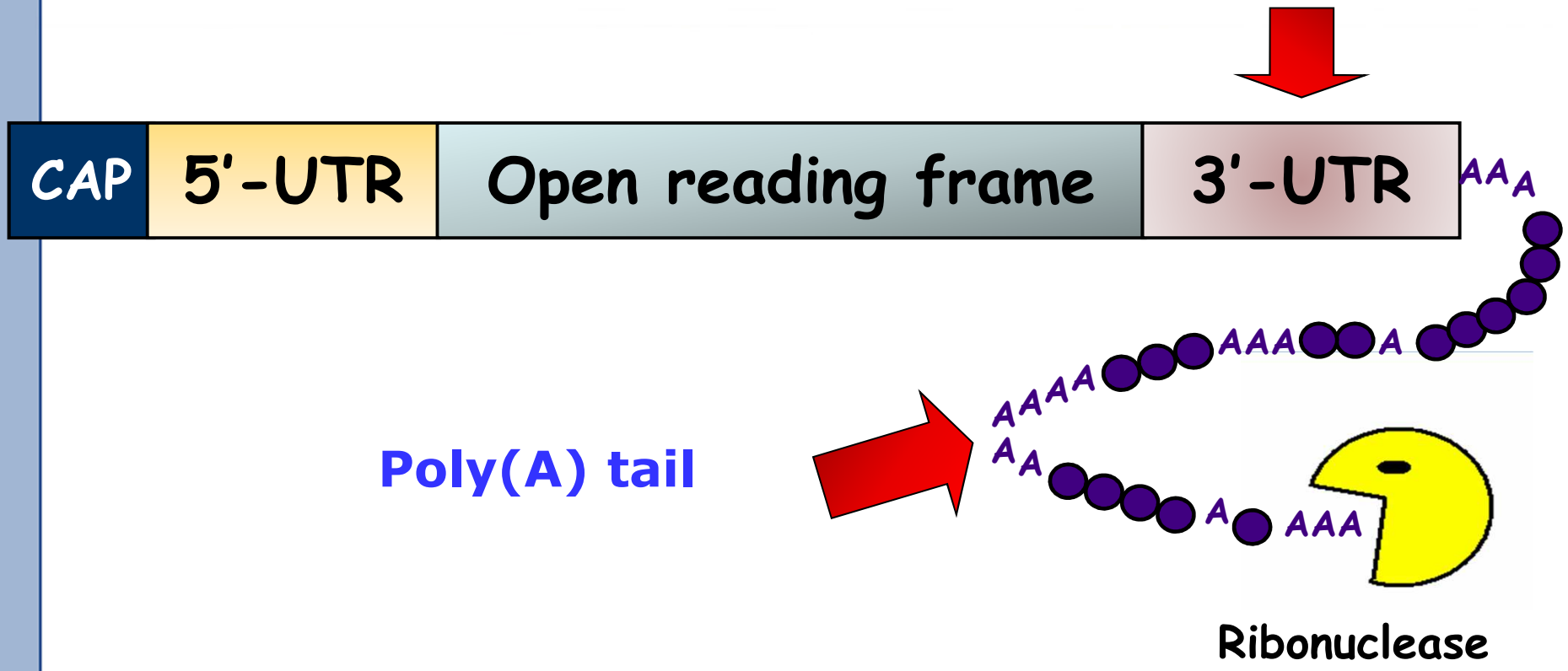
HuR **ubiquitously expressed**



How do ELAV work???

mRNA of a target gene

Adenine and Uracil-rich elements (ARE)



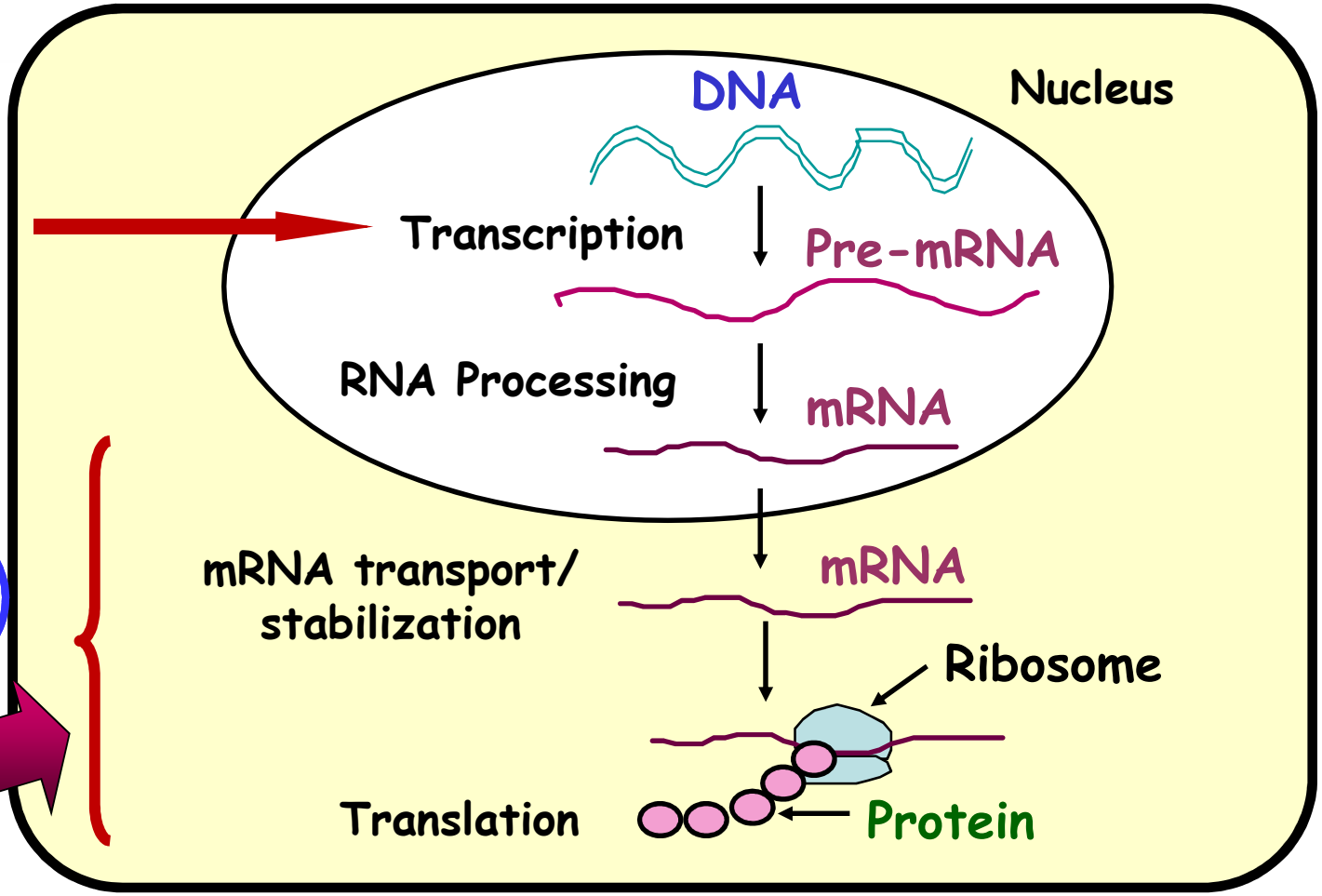
ELAV proteins are highly conserved RNA Binding proteins known to bind preferentially to adenine and uracil-rich elements (AREs) found in the 3'-untranslated region (3'-UTR) of a subset of mRNAs, including those of many early responsive genes, which through this 3'-UTR are targeted for rapid degradation. ELAV proteins have been reported to **mainly act by increasing the cytoplasmic stability and/or rate of translation of ARE-containing mRNAs**.

Gene expression regulation

Transcriptional control

Post-transcriptional control

ELAV



DNA → RNA → Protein

Advantages of a post-transcriptional control

✓ Affect gene expression within a short time.

✓ Allow a localized modification of the protein content in specific subcellular compartments.

Background

✓**Activation of Protein Kinase C (PKC) increases as a consequence of the hyperglycemia associated with diabetes.**

✓**Among the different PKC isoforms, the beta seems to be preferentially activated in the retina.**

✓ **PKC has been involved in the positive control of VEGF expression.**

✓ **VEGF belongs to the 5-8% of human genes bearing in their mRNA a specific *cis* signal able to affect the half-life of the mRNAs themselves.**

These considerations prompted us to investigate whether the ELAV/HuR protein could represent a final target of the signal cascade involving upstream specifically PKC beta and resulting downstream in the stabilization and translation of VEGF mRNA

PKCβ activation

↓

HuR — P

↓

VEGF mRNA-HuR binding

↓

VEGF protein content

ELSEVIER

Pharmacological Research 57 (2008) 60–66

Pharmacological research

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PKCβII/HuR/VEGF: A new molecular cascade in retinal pericytes for the regulation of VEGF gene expression

Marialaura Amadio[a,*], Giovanni Scapagnini[b], Gabriella Lupo[c], Filippo Drago[d], Stefano Govoni[a], Alessia Pascale[a]

[a] *Department of Experimental and Applied Pharmacology, Via Taramelli 14, 27100 Pavia, Italy*
[b] *Department of Health Sciences, University of Molise, Italy*
[c] *Department of Biochemistry, University of Catania, Italy*
[d] *Department of Experimental and Clinical Pharmacology, University of Catania, Italy*

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Collaboration with Proff. Drago and Bucolo

Role of the PKC beta/HuR/VEGF pathway in an "in vivo" model of diabetes and its pharmacological modulation

[Amadio et al., Biochemical Pharmacology 80 1230–1237, 2010]

Animal model
(Prof. Drago's lab)

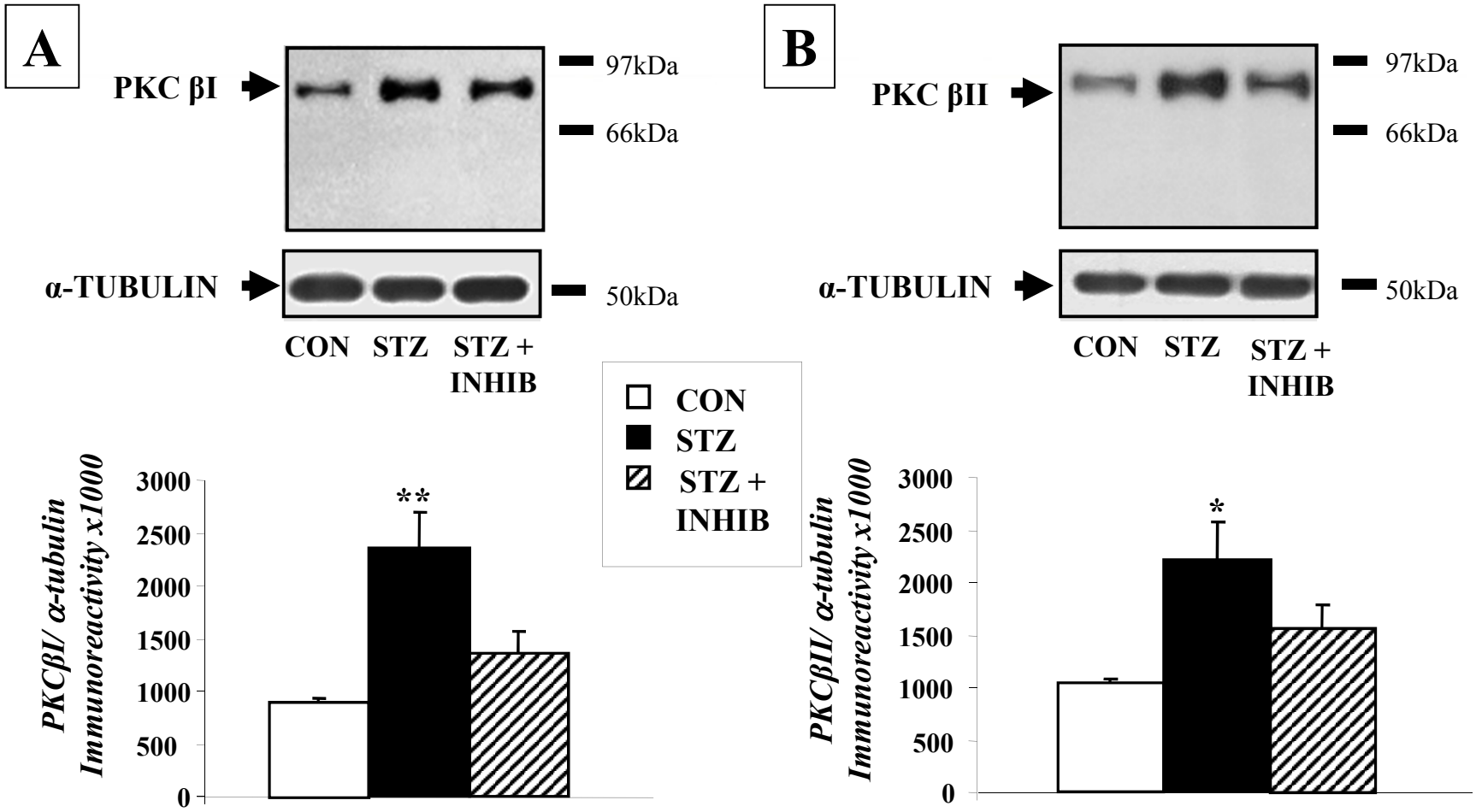
- **Control group (vehicle)**

- **Streptozotocin (STZ) group**:
  Time 0: i.v. administration of STZ (60 mg/kg).

- **STZ + PKC beta inhibitor (LY-379196) group:**
  Time 0: i.v. administration of STZ (60 mg/kg).
  After 30 min: first administration of LY-379196 (1mg/kg i.p.) and once-a-day for the following 10 days

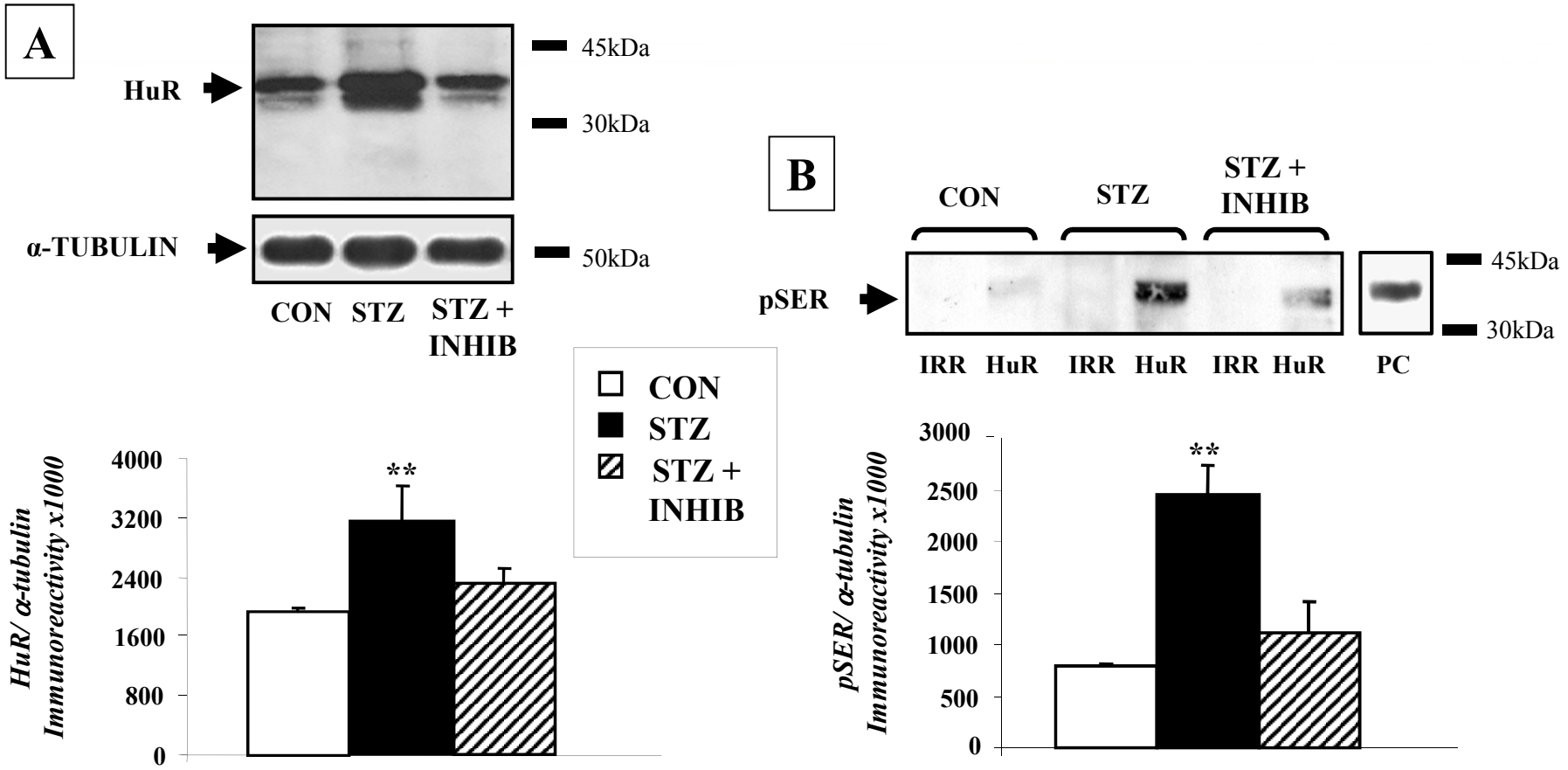
- All the rats were sacrificed on the 10th day after 6 hours from the last administration of the LY-379196

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 (STZ+INHIB) rats. Mean grey level ratios (mean ± 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.

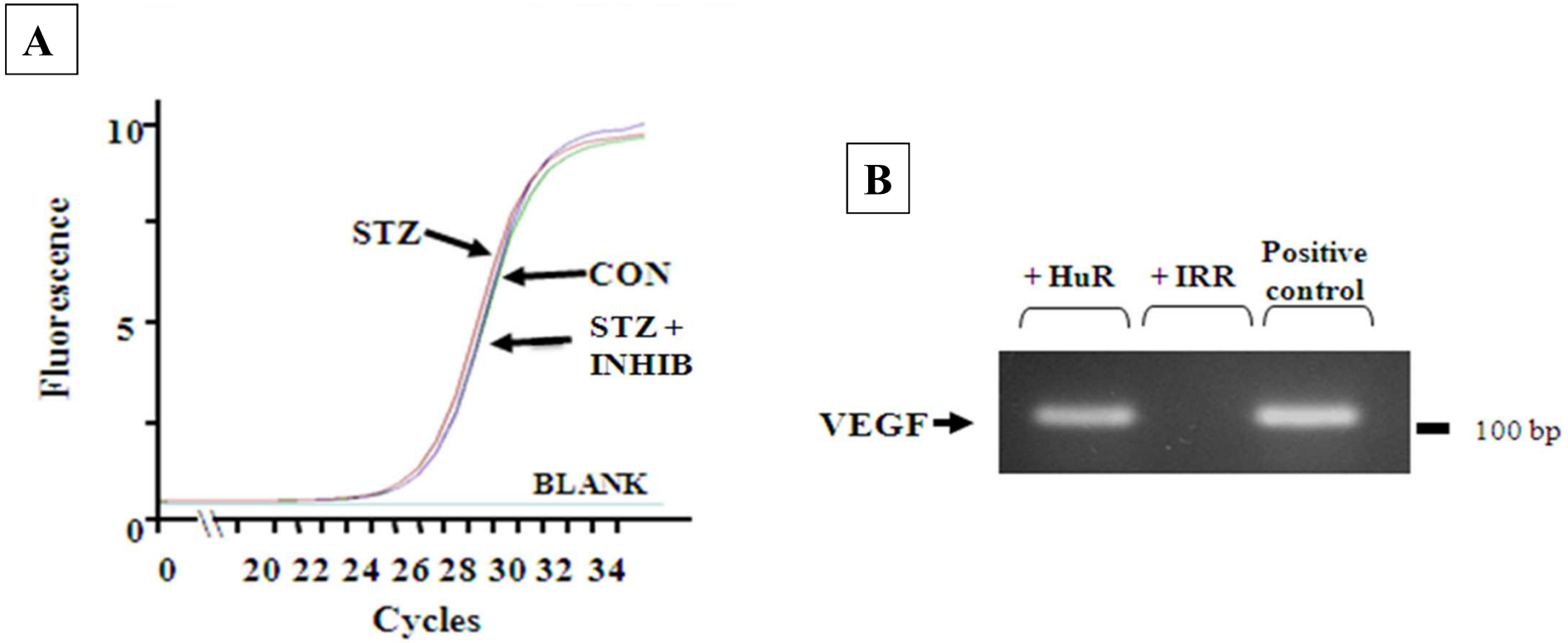
Upregulation and PKC-mediated phosphorylation of HuR in the retina from diabetic rats.



(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 (STZ+INHIB) rats. Mean grey level ratios (mean ± 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 (ISTZ+NHIB) 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 ± 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.

Does HuR protein bind to VEGF mRNA in the retina?

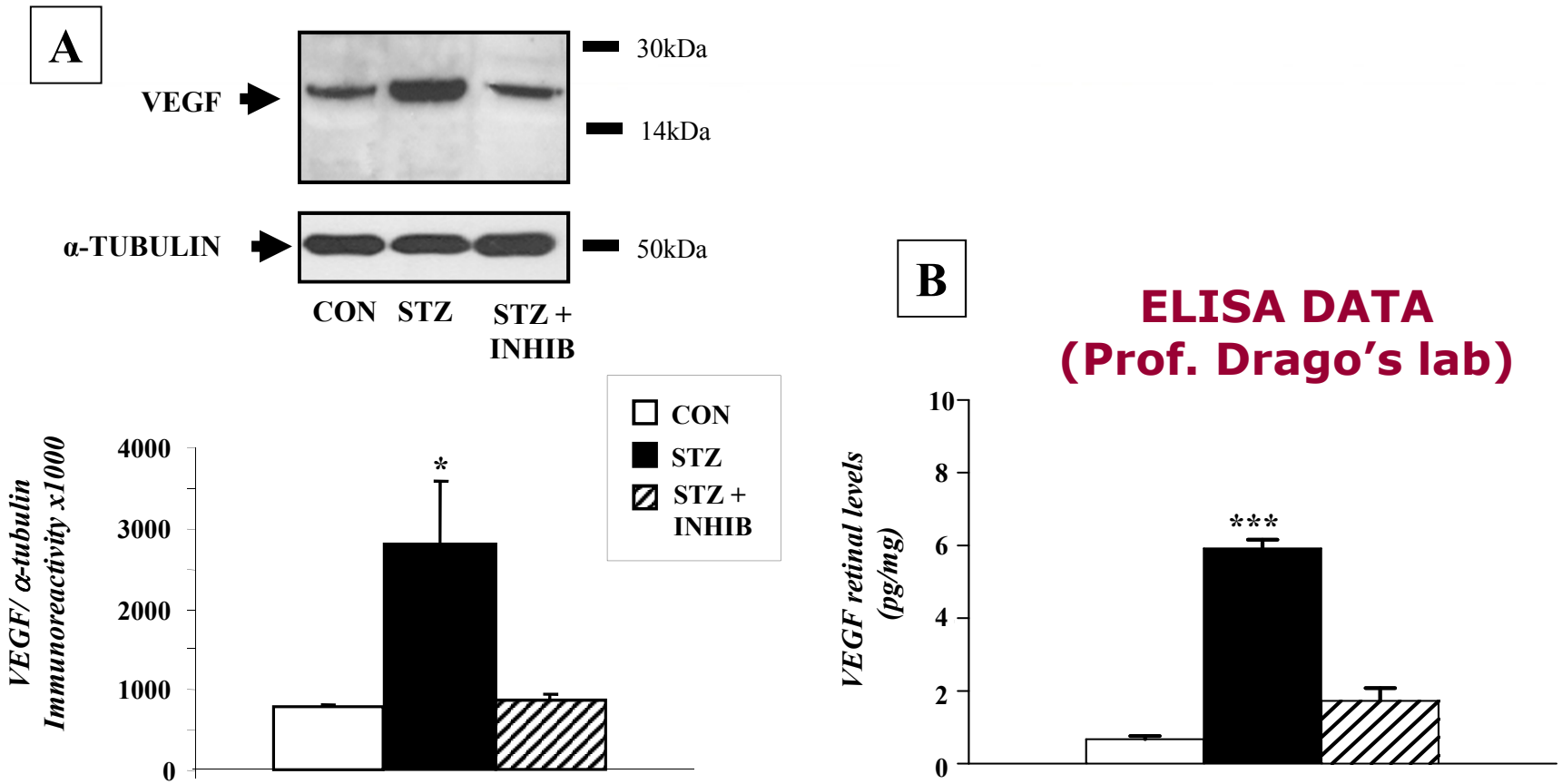
HuR protein binds to VEGF mRNA in the rat retina



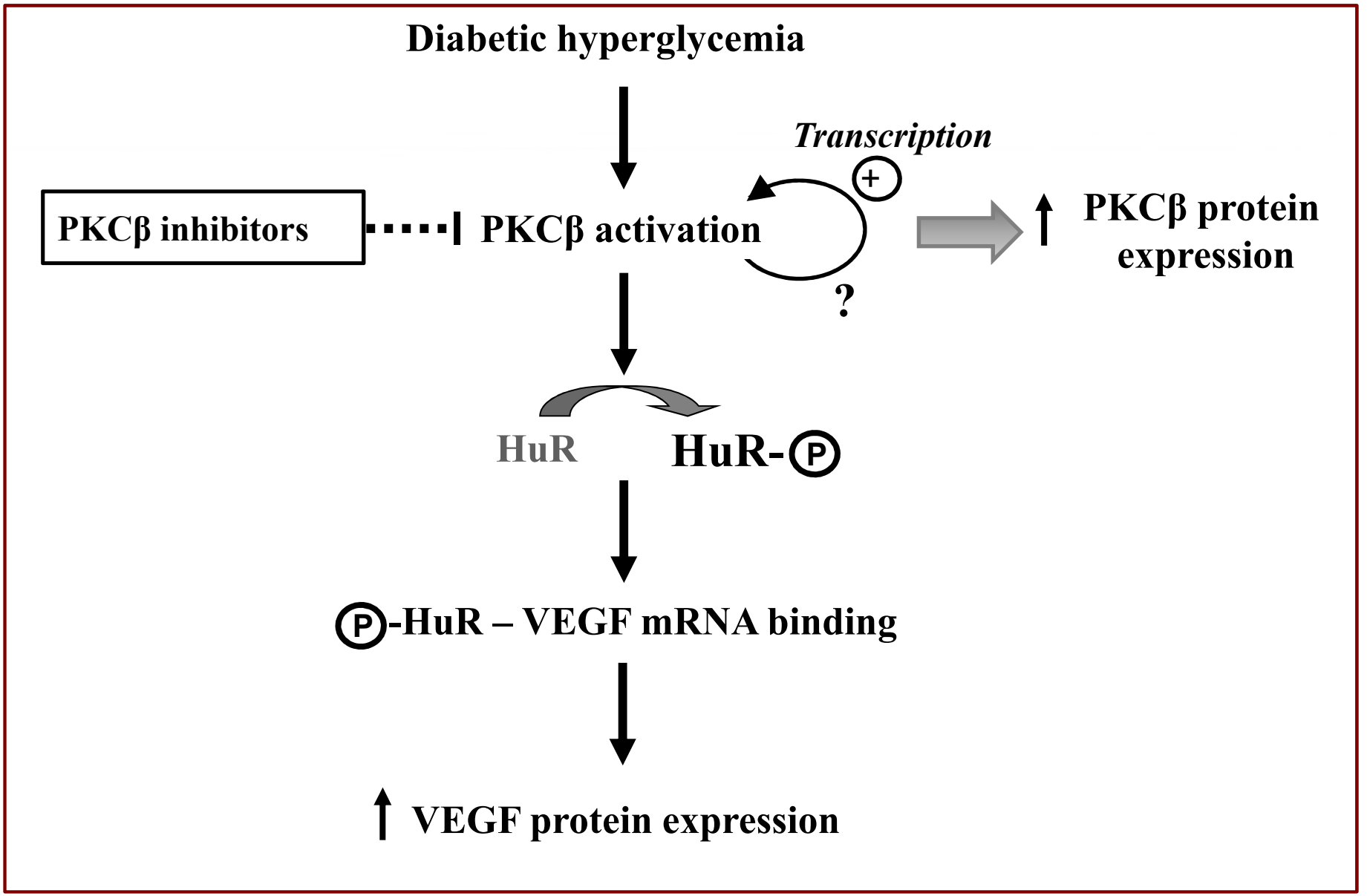
Representative Real Time-PCR amplification plots (**Panel A**) of VEGF in the retina from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (STZ+INHIB) rats. (**Panel B**) Representative experiment showing the control samples following immunoprecipitation experiments using the anti-HuR antibody (+HuR) or an irrelevant antibody (+IRR) with the same isotype of HuR (as a negative control) subjected to RT-PCR and run in an agarose gel. A cDNA obtained from a total mRNA extract was utilized as a positive control.

What happens to VEGF protein expression?

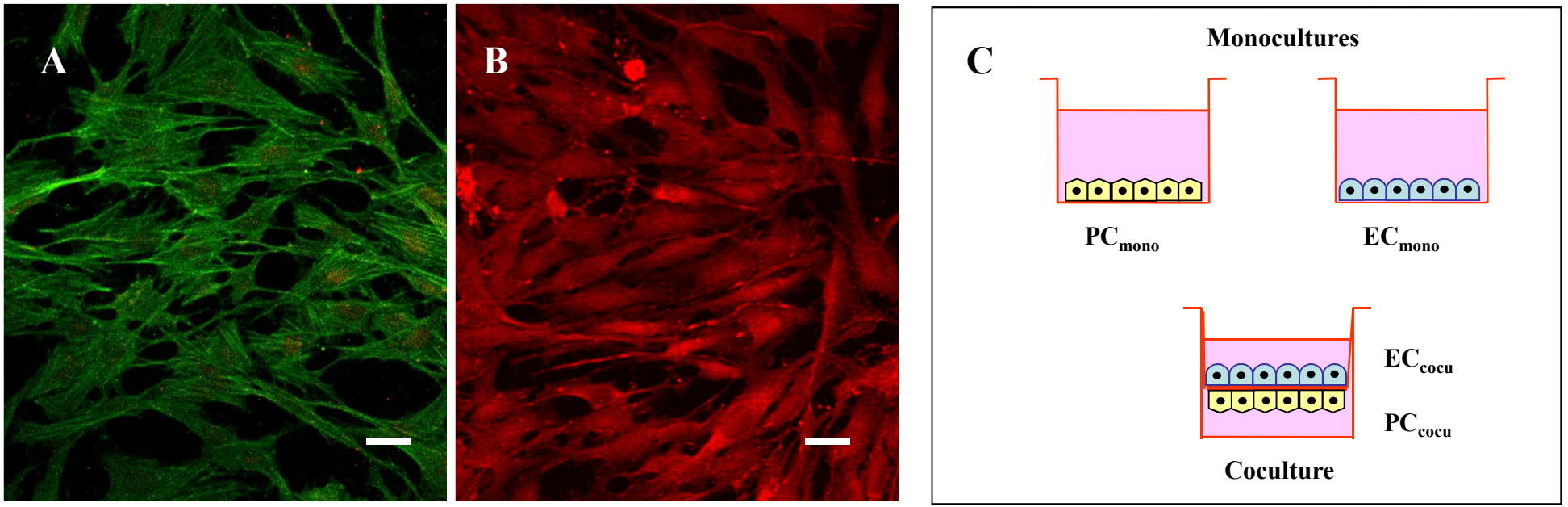
VEGF levels increase in the retina from diabetic rats



(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 (STZ+INHIB) rats. Mean grey level ratios (mean ± 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.


Diabetic hyperglycemia
Transcription
+
PKCβ inhibitors
PKCβ activation
PKCβ protein expression
?
HuR
HuR-P
P-HuR – VEGF mRNA binding
VEGF protein expression

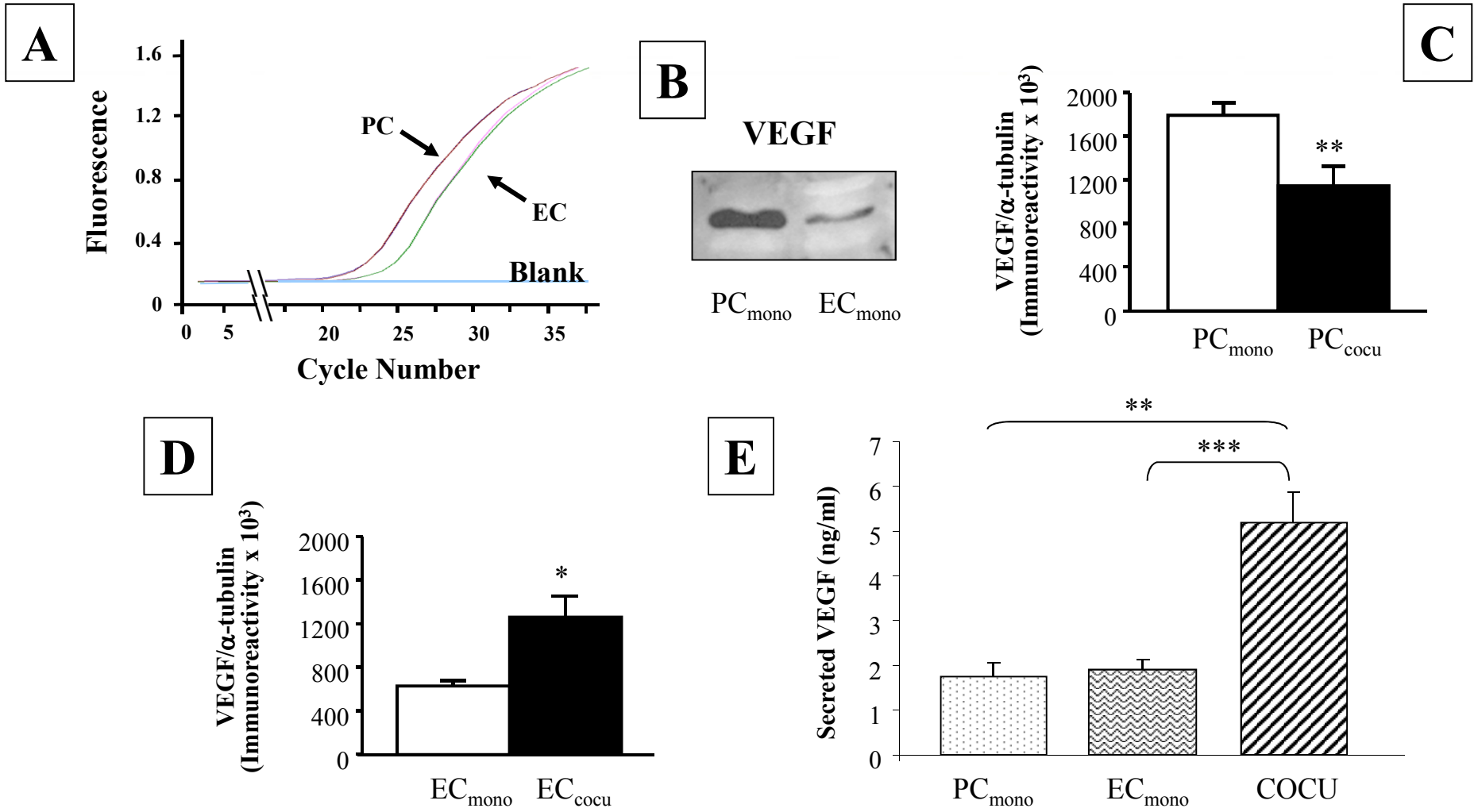
Collaboration with Proff. Drago and Lupo



Monocultures of pericytes and endothelial cells, and cocultures with direct cell-to-cell contact. (**A-B**) Confocal fluorescence representative images of retinal pericytes and endothelial cells in coculture. (**A**) Pericytes monolayer was stained with a monoclonal anti-α-actin antibody coupled to a green fluorescent protein-labeled FITC secondary antibody. (**B**) Endothelial cells monolayer was stained with a polyclonal anti-vWF antibody coupled to a red fluorescent protein-labeled Cy3 secondary antibody. Scale bars: 20 μm. (**C**) Schematic representation of mono- and cocultures.

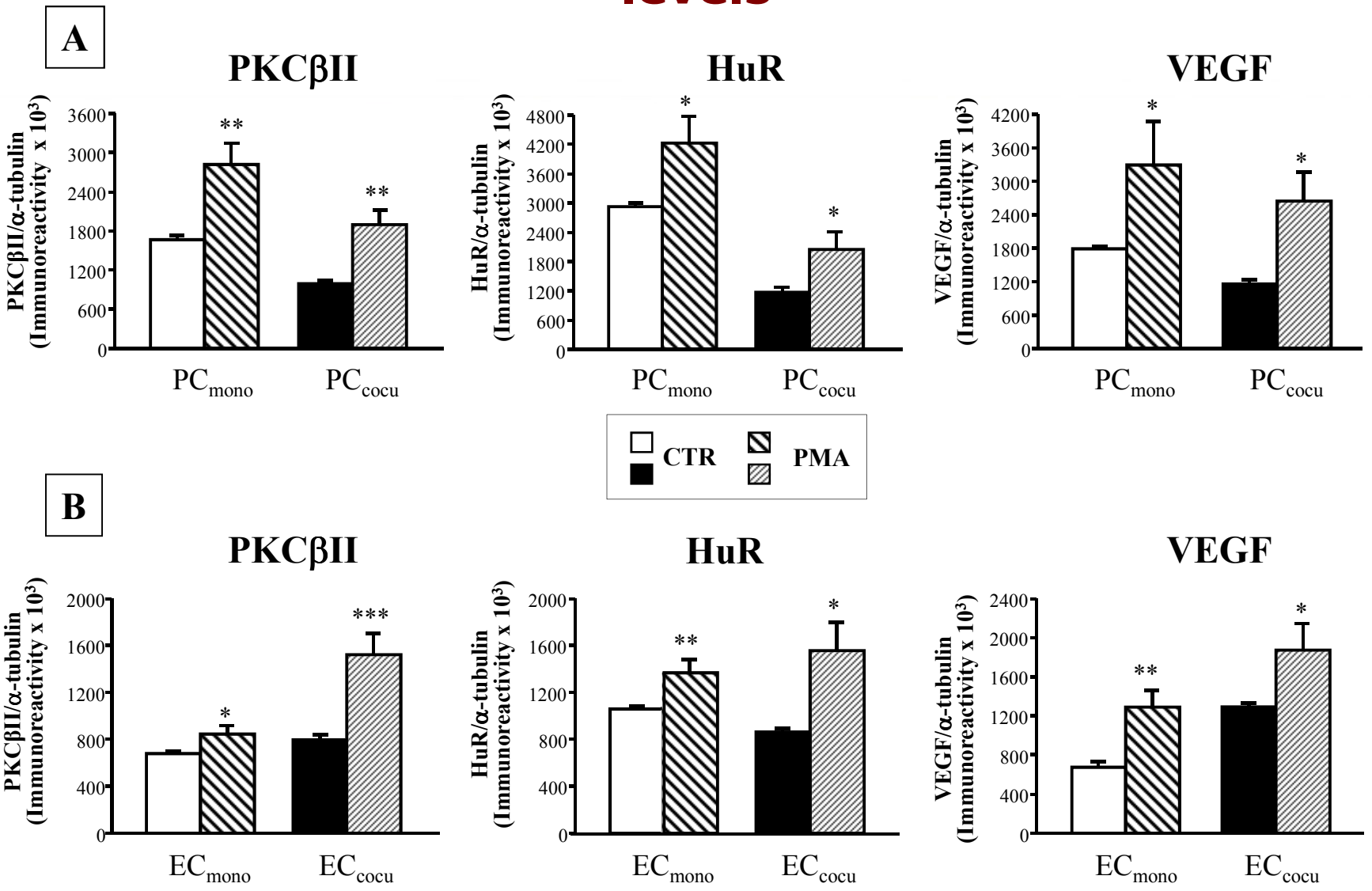
[Amadio et al., Molecular Vision 18:2153-2164, 2012]

VEGF protein levels are affected by culture conditions in both pericytes and endothelial cells.



(**A**) Representative real time RT-PCR amplification plots relative to VEGF mRNA content in pericytes (PC) and endothelial cells (EC) (Ct mean ± S.E.M.: PC: 26.9 ± 1.1; EC: 28.8 ± 1.7). (**B**) Representative Western blotting of VEGF protein in the total homogenates of pericytes and endothelial cells cultured separately (PC_{mono} and EC_{mono}, respectively). (**C-D**) Mean grey levels ratios (mean ± S.E.M.) of VEGF/α-tubulin immunoreactivities measured by Western blotting in PC (**C**) and EC (**D**). All the comparisons were performed between cells in monoculture ($_{mono}$) and cells in coculture ($_{cocu}$). *p<0.05; **p<0.01, n=4. (**E**) VEGF protein levels measured in cell culture conditioned media of PC_{mono}, EC_{mono} and coculture. **p<0.01; ***p<0.001, n=4, Tukey-Kramer test.

PMA treatment increases PKCβII, HuR, and VEGF protein levels



(**A-B**) Mean grey levels ratios (mean ± S.E.M.) of PKCβII/α-tubulin, HuR/α-tubulin, VEGF/α-tubulin immunoreactivities measured by Western blotting in pericytes (PC; **A**) and endothelial cells (EC; **B**). The comparisons were performed between control (CTR) and PMA-treated (PMA) cells in monoculture ($_{mono}$) and in coculture ($_{cocu}$) separately. *p<0.05; **p<0.005, n=5.

CONCLUSIONS

The PKCβ/HuR/VEGF pathway is activated *in vivo* in the retina from diabetic rats:

- ✓ **PKCβ triggers an increase of HuR expression and phosphorylation causing an augmentation in VEGF protein levels**

Data from pericytes/endothelial cells cocultures:

- ✓ **The results suggest that PKC activation (as in the early stage of Diabetic Retinopathy) induces changes in PKCβ/HuR/VEGF cascade protein expression in both pericytes and endothelial cells.**
- ✓ **Further studies on pericytes/endothelial cells may help to better understand Diabetic Retinopathy pathogenesis**

The PKCβ/HuR/VEGF cascade may represent a potential pharmacological target useful to counteract pathologies implicating VEGF deregulation, such as diabetic retinopathy

Dipartimento di Scienze del Farmaco, Sez. di Farmacologia Pavia

- **Prof. Stefano Govoni**
- **Dr. Marialaura Amadio**
- **Dr. Cecilia Osera**

Dip. di Biomedicina Sperim.e Clinica, Sez.Farmacologia e Biochimica, Università di Catania

- **Prof. Filippo Drago**
- **Prof. Claudio Bucolo**
- **Dr. Gianmarco Leggio**

Dipartimento di Biochimica, Università di Catania

- **Prof. Gabriella Lupo**
- **Dr. Carla Motta**

..... AND FOR YOUR ATTENTION ☺!!!

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The PKCβ/HuR/VEGF pathway in diabetic retinopathy

M. Amadio [a,1], C. Bucolo [b,1,*], G.M. Leggio [b], F. Drago [b], S. Govoni [a], A. Pascale [a]

[a] Department of Experimental and Applied Pharmacology, Centre of Excellence in Applied Biology, University of Pavia, Pavia, Italy
[b] Department of Experimental and Clinical Pharmacology, University of Catania, Catania, Italy

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Protein kinase C activation affects, via the mRNA-binding Hu-antigen R/ELAV protein, vascular endothelial growth factor expression in a pericytic/endothelial coculture model

M. Amadio,[1] C. Osera,[1] G. Lupo,[2] C. Motta,[2] F. Drago,[3] S. Govoni,[1] A. Pascale[1]

[1]Department of Drug Sciences, Section of Pharmacology, University of Pavia, Pavia, Italy; [2]Department of Biochemistry, University of Catania, Catania, Italy; [3]Department of Clinical and Experimental Biomedicine, Section of Pharmacology and Biochemistry, University of Catania, Catania, Italy

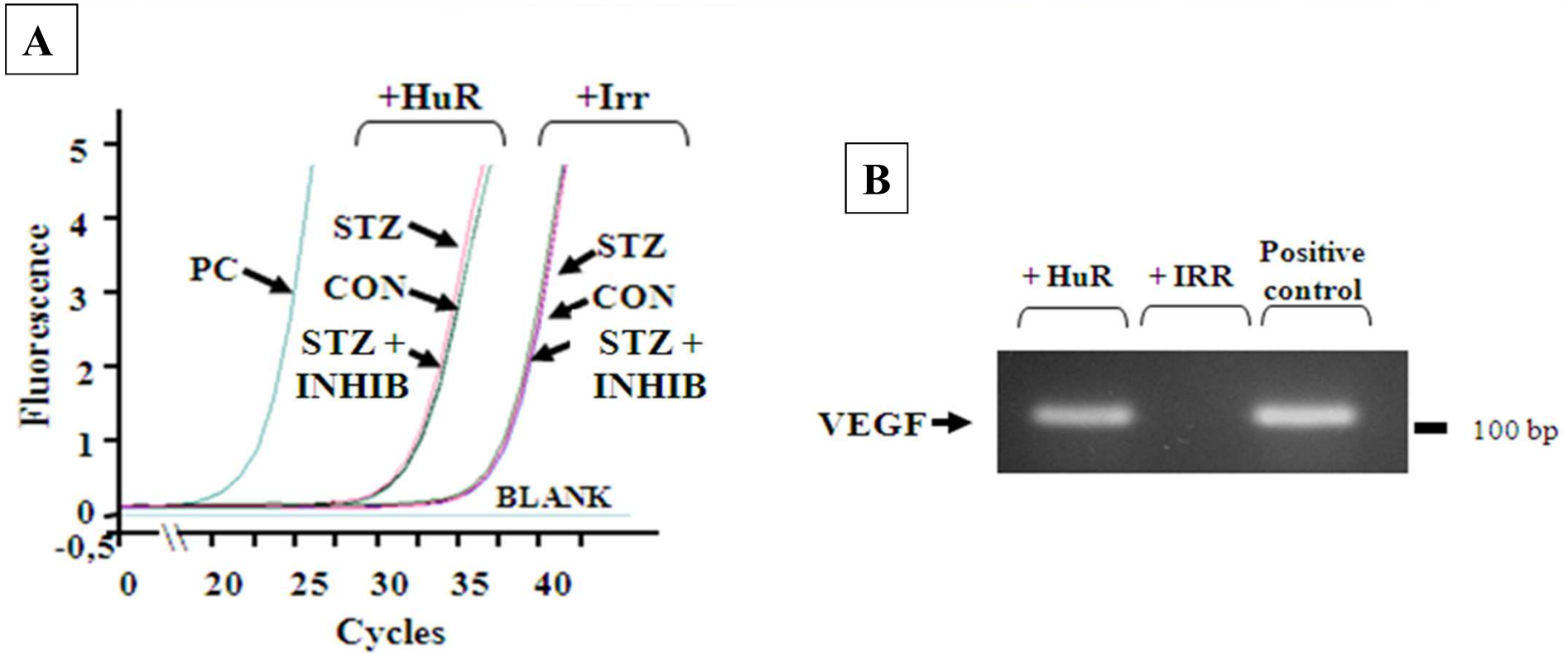
Effects of STZ-induced diabetes on body weight and blood glucose levels in different groups after ten days.
(Prof. Drago's lab)

Groups	Body weight (g)	Nonfasting blood glucose (mg/dl)
Control (non-diabetic)	240 ± 21	99 ± 14
Diabetic	185 ± 16	408 ± 23*
Diabetic + LY379196	190 ± 18	399 ± 40

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

Table 1. Control group are normal rats injected with the vehicle used to dissolve STZ. LY379196, a selective inhibitor of PKCβ, was given at 1mg/kg (i.p.) per day. Diabetes was induced by 60 mg/kg (i.v.) injection of STZ.

HuR protein binds to VEGF mRNA in the rat retina



Representative Real Time-PCR amplification plots (**Panel A**) of VEGF in the retinal mRNPs from control (CON), streptozotocin-induced diabetic (STZ) and STZ plus LY379196 (STZ+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 B**) 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.

TABLE 1. TRANS ENDOTHELIAL ELECTRICAL RESISTANCE (TEER) AND PERMEABILITY TO SODIUM FLUORESCEIN (FL P_{e}^{E}) ON ENDOTHELIAL CELLS IN MONOCULTURE (EC_{MONO}) AND IN COCULTURE WITH PERICYTES (EC_{COCU}).

Cell culture condition	TEER (W × cm^2)	FL Pee (10^{-6} cm/s)
ECmono	90±22.0	6.6±0.5
ECcocu	230±56.3 *	3.9±0.21 *

EC (40,000 cells/cm^2) were cultured in monolayers or were grown on the top surface of the Transwell insert (6-well type, 0.4-μm pore size) in which PC (40,000 cells/cm^2) were first plated on the outside of the polycarbonate membrane. After 24 h co-incubation, measurements of TEER and Permeability on EC were performed as described in Materials and Methods. Values (means±S.E.M.) are from three independent experiments. * $p<0.01$.

TABLE 2. PKCβI, PKCβII AND HuR PROTEIN LEVELS IN THE TOTAL HOMOGENATES OF PERICYTES (PC) AND ENDOTHELIAL CELLS (EC).

Protein	Cell type/culture condition	Immunoreactivity
PKCβI	EC_{mono}	819.0±125.9
	EC_{cocu}	708.0±79.6
PKCβII	PC_{mono}	1631.3±29.2
	PC_{cocu}	998.7±149.1 *
	EC_{mono}	646.7±45.4
	EC_{cocu}	765.4±119.1
HuR	PC_{mono}	2802.1±78.4
	PC_{cocu}	1211.5±94.4 **
	EC_{mono}	1040.4±64.4
	EC_{cocu}	897.8±181.4

The values are expressed as mean gray levels ratios ±SEM of PKCβI/α-tubulin, PKCβII/α-tubulin, HuR/α-tubulin immunoreactivities measured by western blotting in PC and EC. The comparisons were performed between the same cell type cultured in monoculture ($_{mono}$) and coculture ($_{cocu}$). * $p<0.001$; ** $p<0.0001$; n=5.

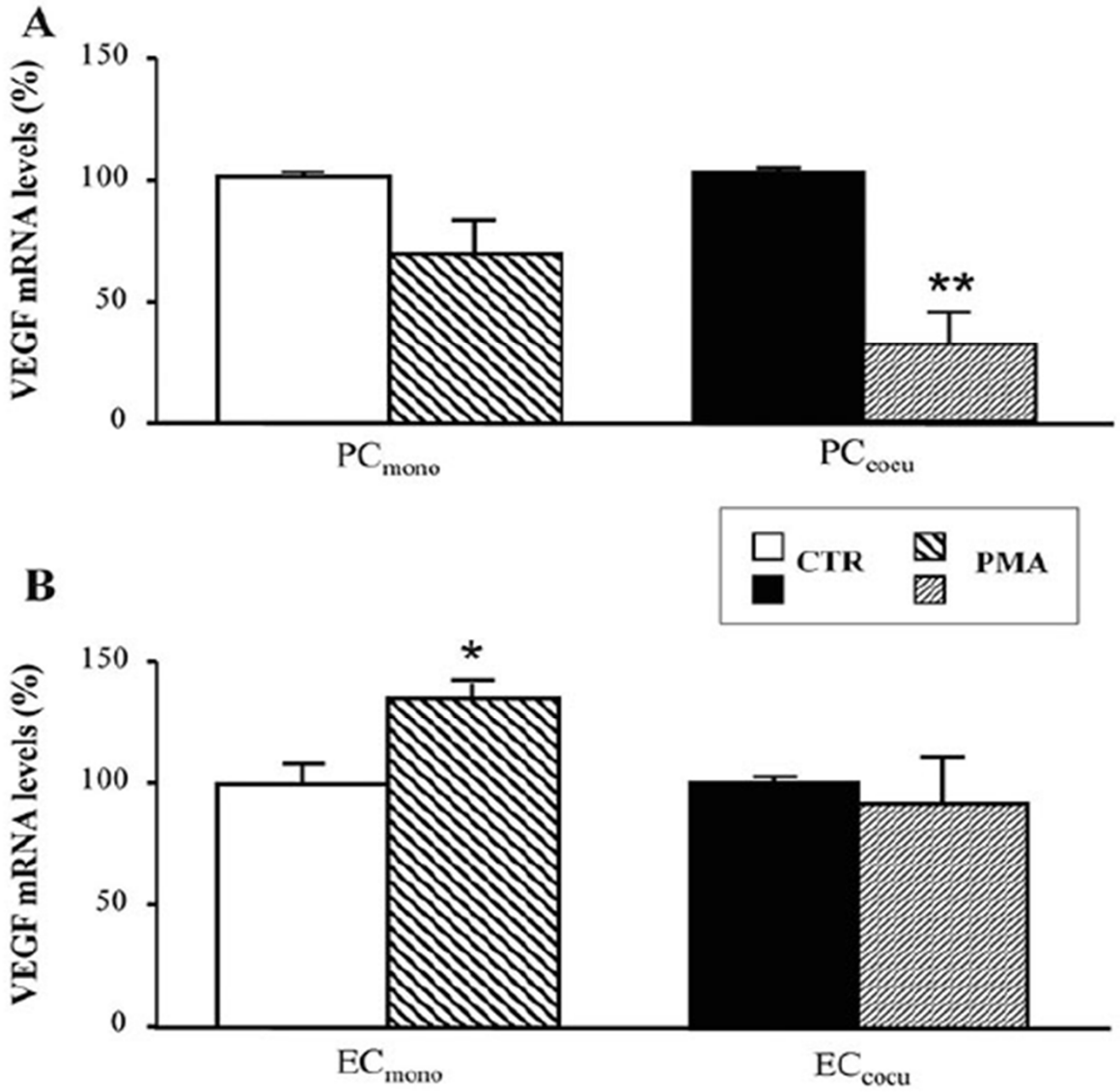
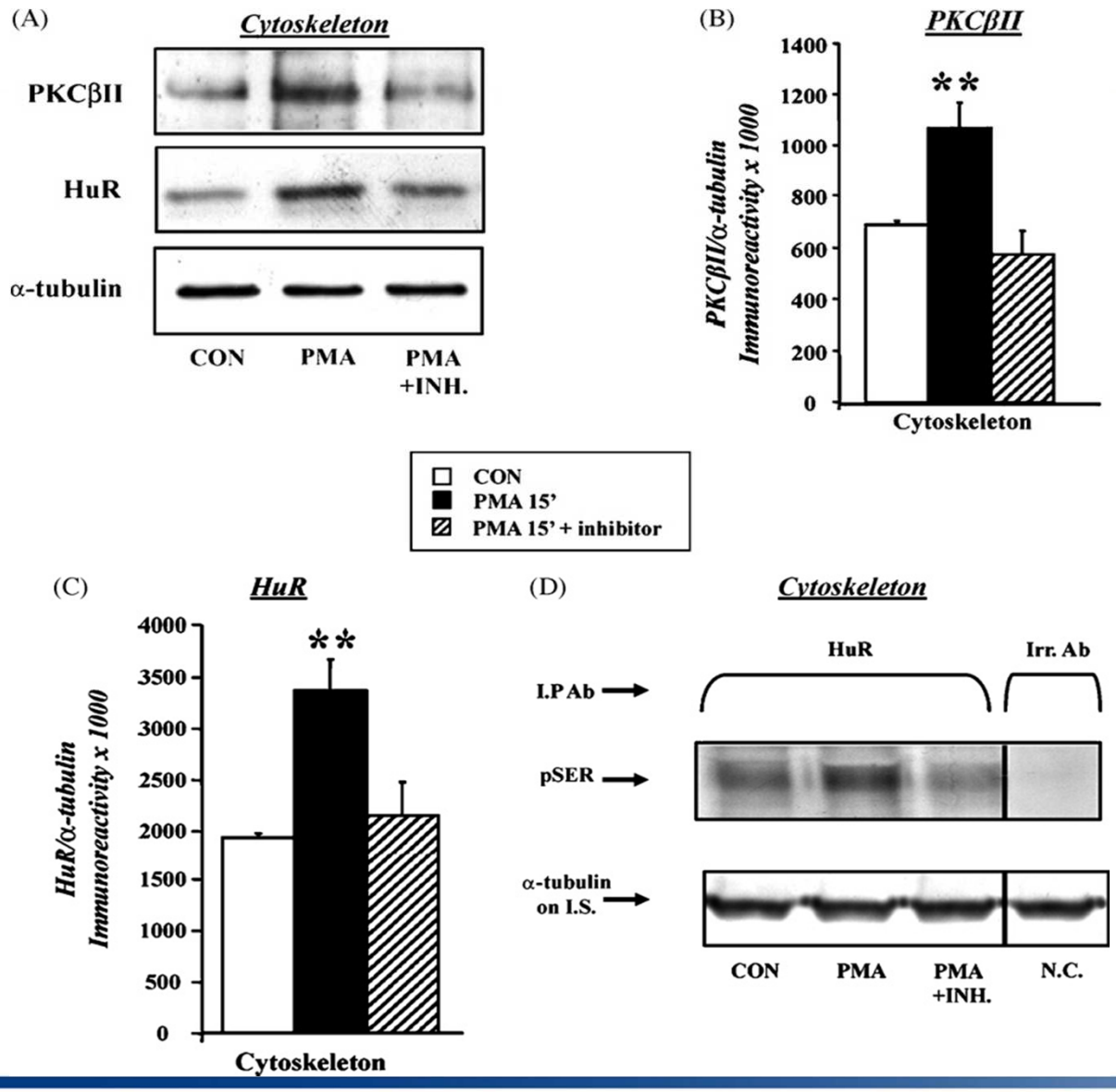


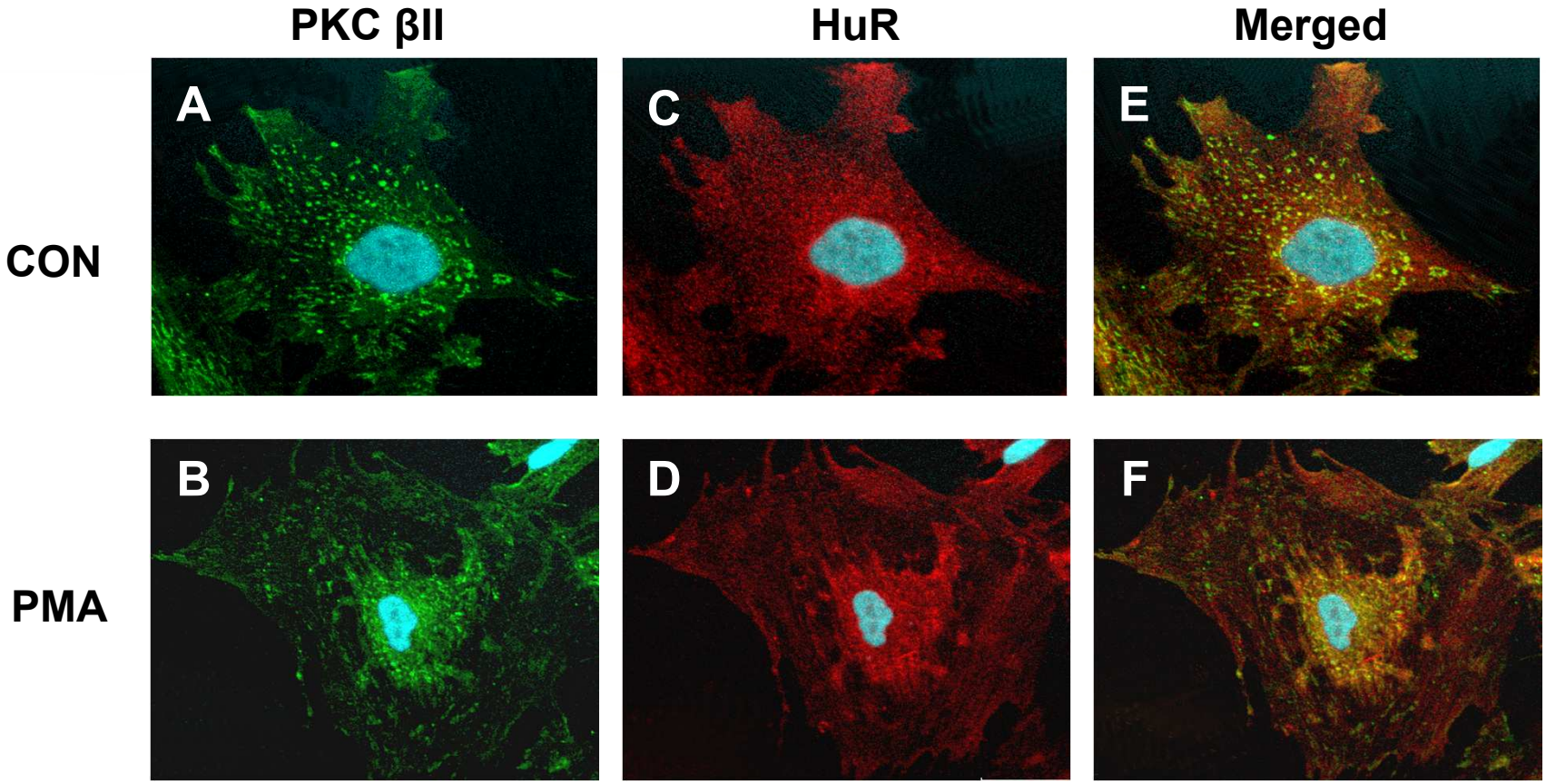
Figure 5. The changes in *VEGF* mRNA levels induced by PKCβ activation depend on the cell type and the culture condition. **A-B**: Determination of the levels of *VEGF* mRNA with quantitative real time RT–PCR in the pericytes (PC; **A**) and endothelial cells (EC; **B**). The measures of the total *VEGF* mRNA were normalized in accordance with the corresponding levels of *RPL10a* mRNA. The values are expressed as mean±SEM. The comparisons were performed between control (CTR) and PMA-treated (PMA) cells in monoculture ($_{mono}$) and in coculture ($_{cocu}$). The experiments were performed on three distinct sets of cells. *$p<0.01$; **$p<0.005$, n=5.

PKCβII and HuR belong to the same molecular cascade in retinal pericytes



(A) Representative Western blottings of PKCβII, HuR and α-tubulin in the cytoskeleton from control (CON), PMA (15 minutes at 100 nM) and PMA plus LY379196 PKCβII inhibitor (INH; at 30nM concentration) treated retinal bovine pericytes. (B-C) Mean grey levels ratios (mean ± S.E.M.) of PKCβII/α-tubulin (B) and HuR/α-tubulin (C) immunoreactivities measured by Western blotting in the cytoskeleton from control, PMA and PMA plus LY379196 PKCβII inhibitor treated retinal bovine pericytes. **p<0.01, Dunnett Multiple Comparisons test, n=5-6. (D) Representative western blots of phosphorylated serine (pSer) residues present in HuR protein in the cytoskeletal fractions of control, PMA and PMA plus LY379196 PKCβII inhibitor treated cells. For immunoprecipitation experiments an irrelevant isotype-matched IgG (Irr. Ab) has been used as negative control (N.C.). The samples were normalized according to α-tubulin values measured on the input signals (I.S.)

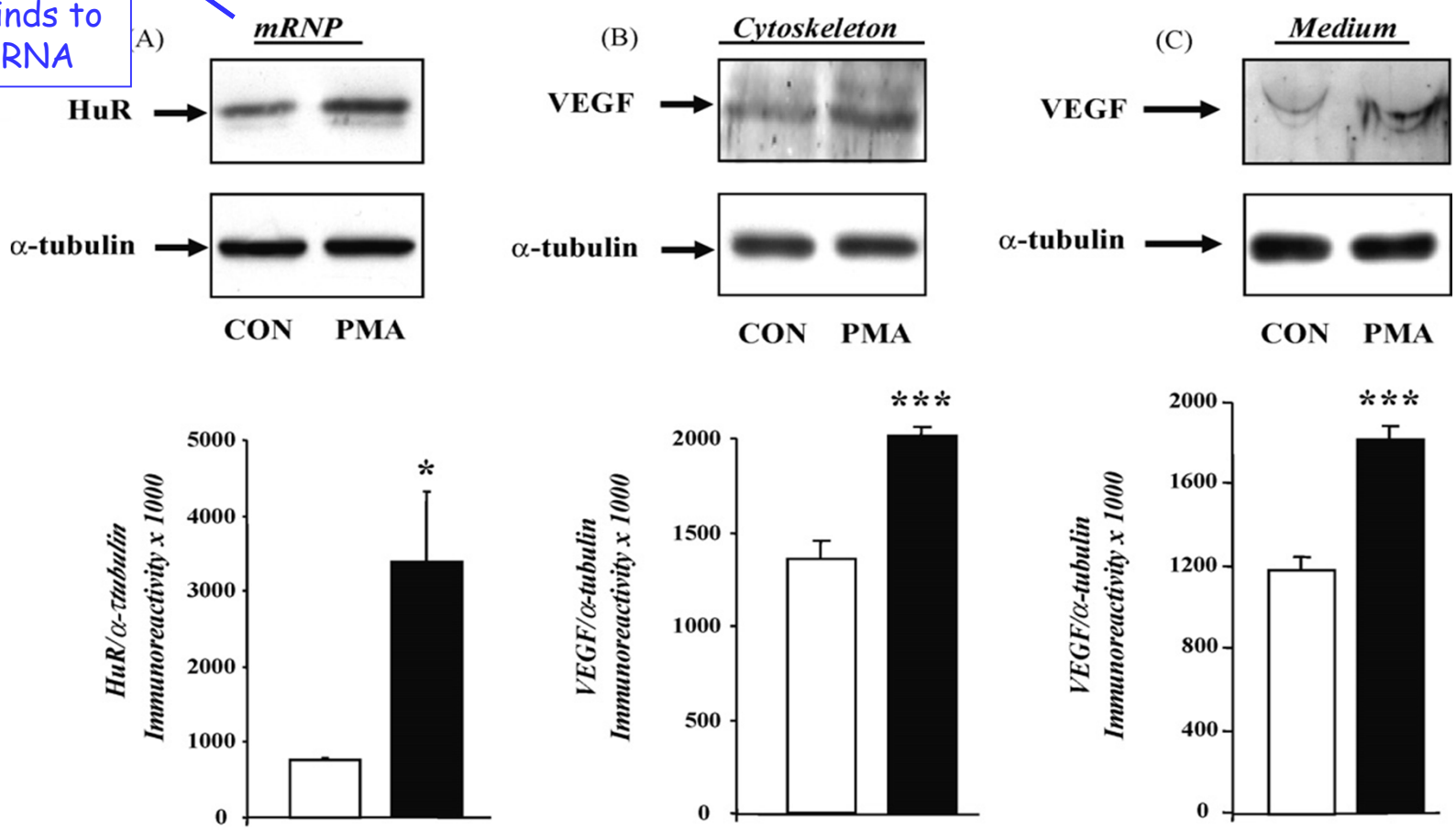
PKCβII and HuR colocalize following PMA exposure



Confocal fluorescence representative images of control (CON) or PMA (15 minutes at 100 nM) treated retinal pericytes. PKCβII (green) and HuR (red) stainings are concentrated around the nuclear region in treated cells (as indicated by yellow dots in panel F) while they are mostly spread all around the cytoplasm in control cells. Nuclei are stained with DAPI (blue). Scale bar: 25 μm.

PMA induces an increase of HuR in mRNP and of VEGF both in the cytoskeleton and in the medium

In mRNP HuR protein binds to VEGF mRNA



(**A**) Representative Western blottings of HuR and α-tubulin in mRNP from control (CON) and PMA (15 minutes at 100 nM) treated retinal bovine pericytes (upper). Mean grey levels ratios (mean ± S.E.M.) of HuR/α-tubulin immunoreactivities measured by Western blotting in mRNP from control and PMA treated retinal bovine pericytes (lower). *p<0.05; Student *t*-test, n=5. (**B**) Representative Western blottings of VEGF and α-tubulin in the cytoskeleton from control (CON) and PMA (15 minutes at 100 nM) treated retinal bovine pericytes (upper). Mean grey levels ratios (mean ± S.E.M.) of VEGF/α-tubulin immunoreactivities measured by Western blotting in the cytoskeleton from control and PMA treated retinal bovine pericytes (lower). ***p<0.0001; Student *t*-test, n=5. **(C)** Representative Western blottings of VEGF released in the medium and α-tubulin in the total cellular homogenates from control (CON) and PMA (15 minutes at 100 nM) treated retinal bovine pericytes (upper). Mean grey levels ratios (mean ± S.E.M.) of VEGF immunoreactivities measured by Western blotting in the medium from control and PMA treated retinal bovine pericytes. The VEGF signals were normalized according to α-tubulin content in the plated cells. *p<0.0001; Student *t*-test, n=4 (lower).