The Role of NMDA-receptor in the Regulation of Homocysteine-induced TIMP-1 expression
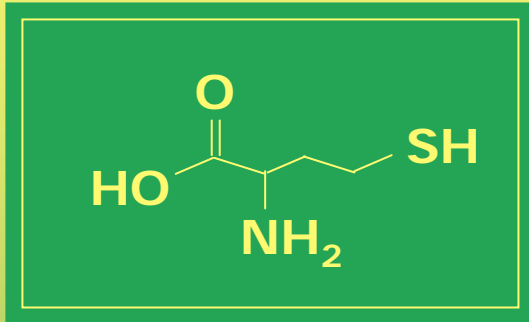
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Summer Program for Undergraduate Research 2007

10 August 2007

Homocysteine

Homocysteine

- amino acid in the blood

- been shown to promote endothelial dysfunction, the formation of the extracellular matrix (ECM), and the sclerotic process

- stimulates the formation of tissue inhibitor of metalloproteinase-1 (TIMP-1) in renal mesangial cells

- TIMP-1 is the most potent inhibitor of the TIMP family, and has been found to be the primary inhibitor of matrix metalloproteinase (MMP) activity

- MMPs are responsible for breaking down collagen and regulating the formation and deposition of the ECM
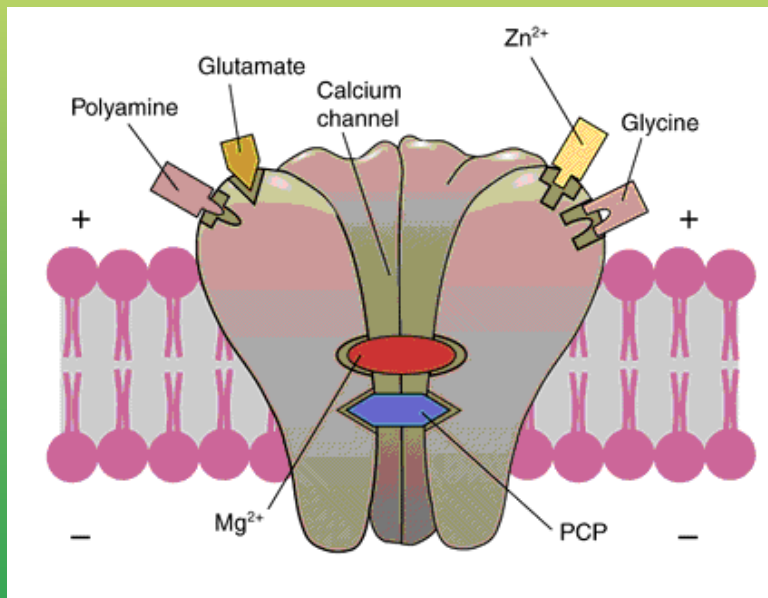
Hyperhomocysteinemia

- a critical pathogenic factor in the progression of end-stage renal disease (ESRD) and in the development of cardiovascular complications related to ESRD

- 85-100% of patients in ESRD also suffer from hyperhomocysteinemia

- even with hemodialysis, patients are still unable to recover

- laboratory studies from animal models

Homocysteine Levels

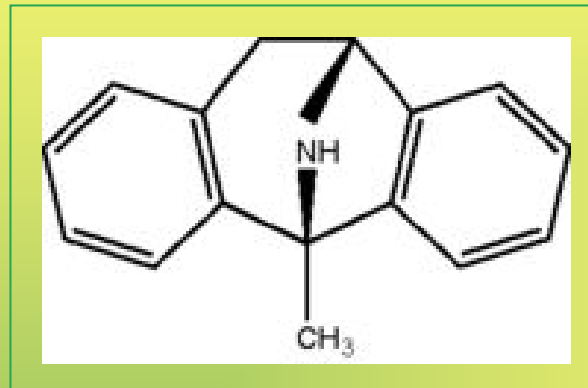|	
< 10 µmol/L	Normal
15-20 µmol/L	Mild
30-60 µmol/L	Moderate
> 80 µmol/L	Severe

N-methyl D-aspartate (NMDA)-receptor

- ionotropic neurotransmitter receptor and ligand-gated nonselective cation channel

- regulated by glutamate and glycine, which are both required for the channel activation and function

- when activated, channel will depolarize and remove the Mg^{2+} block, allowing for an influx of Ca^{2+} ions

- **within some kinds of cells, NMDA receptors play a role in homocysteine uptake**

NMDA-receptor inhibitor MK801 (Dizocilpine)

- non-competitive antagonist of the NMDA receptor

- binds to the inside of the receptor and prevents the flow of ions, including Ca^{2+}

- channel must be open, and this blockage inhibits drugs from binding

Proposed Mechanism

- homocysteine enters the cell through NMDA-receptors

- elevated homocysteine levels increase TIMP-1 levels, causing a decrease in MMP activity

- decrease in MMP activity means collagen and other ECM cannot be cleaved

- formation and deposition of ECM develops into glomerular injury associated with diabetes and hypertension

Hypothesis

NMDA-receptors serve as the pathway for elevated levels of homocysteine to enhance TIMP-1 expression and suppress MMP activity, thereby leading to deposition of the ECM, which is the cause for glomerular injury associated with diabetes and hypertension.

Experimental Design

Real-time RT-PCR

used to measure the TIMP-1 mRNA level

Western Blot Analysis

used to measure the TIMP-1 protein level

Real time RT-PCR

- method for amplifying a specific portion of an RNA molecule

- two major components:
    1) reverse transcription of RNA strand into complementary DNA (cDNA)
    2) exponential amplification of cDNA using polymerase chain reaction

- amplification is made possible through temperature-mediated DNA polymerase

- DNA primers are added, and the iCycler RT-PCR Machine manipulates temperature to bind the sequence specific primers to the cDNA, make double-stranded DNA, and separate the two strands

- the cycle repeats until million of duplicates are formed

- highly sensitive technique, careful pipetting required
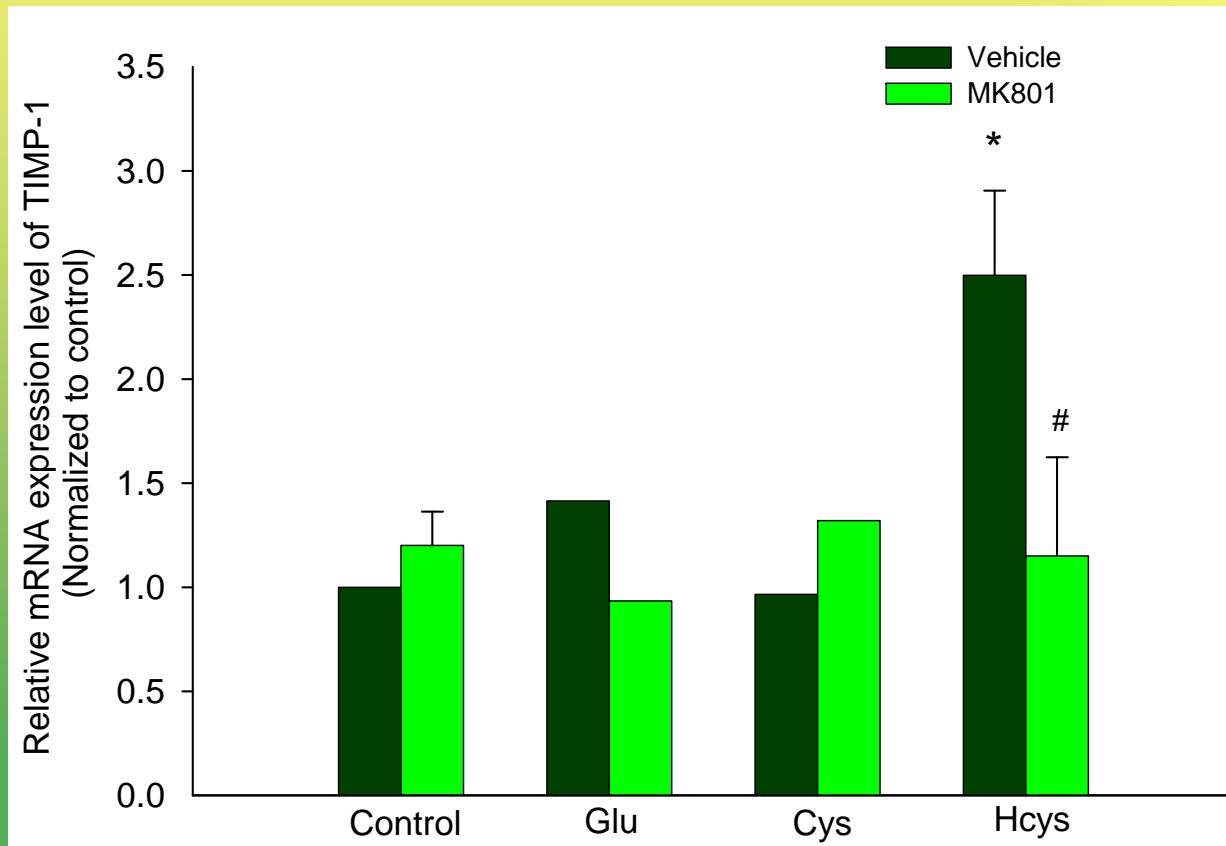
Real time RT-PCR Procedure

Protocol

- culture and treatment of mesangial cells

- RNA Isolation

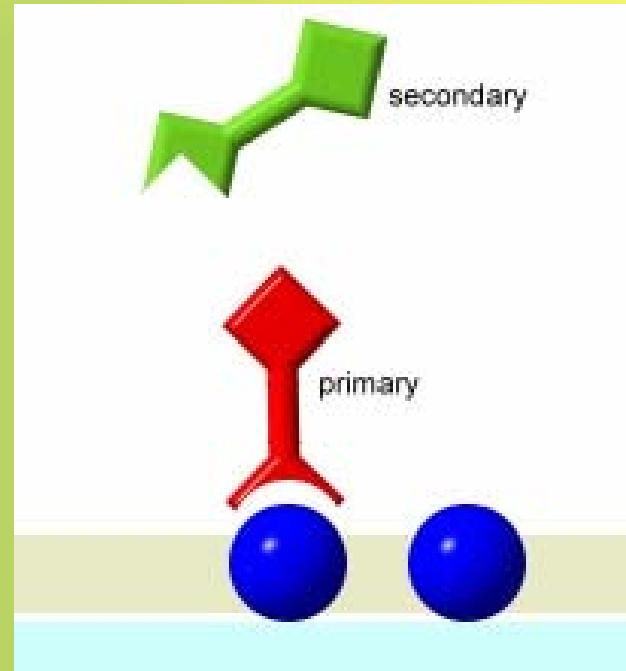- synthesis of cDNA

- real-time RT-PCR

Effect of NMDA-receptor Inhibitor MK801 on the mRNA Level of TIMP-1
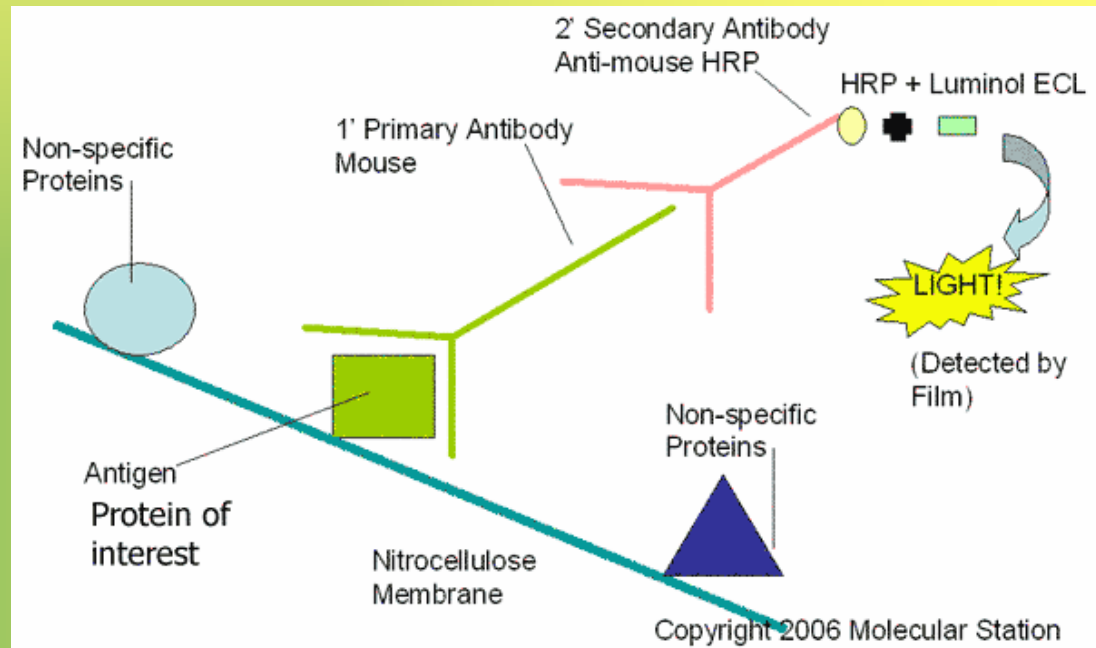
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Western Blot Analysis

- alternately called immunoblot

- method for detecting a specific protein of interest from a great mixture of proteins

- gives information about size and expression amount of interest protein

- protein-specific 1° antibody

- species-specific 2 ° antibody
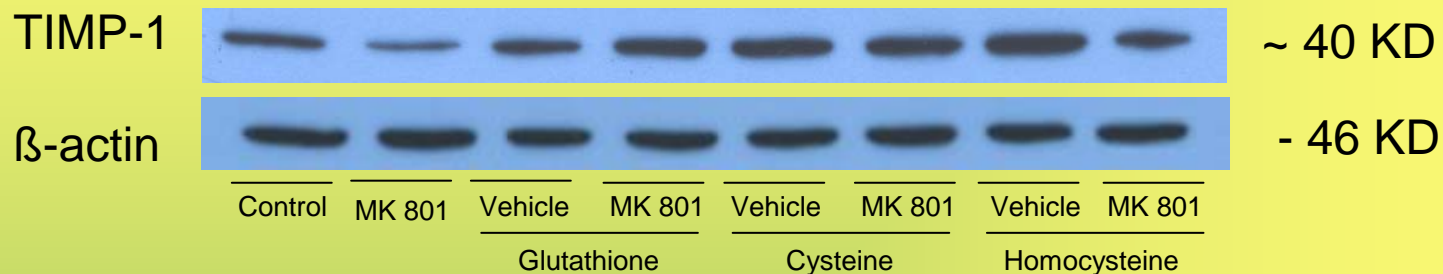
Western Blot Procedure

Protocol

- culture and treatment of mesangial cells

- gel electrophoresis

- transfer from gel to membrane

- membrane blocking

- incubation of 1° and 2° antibodies
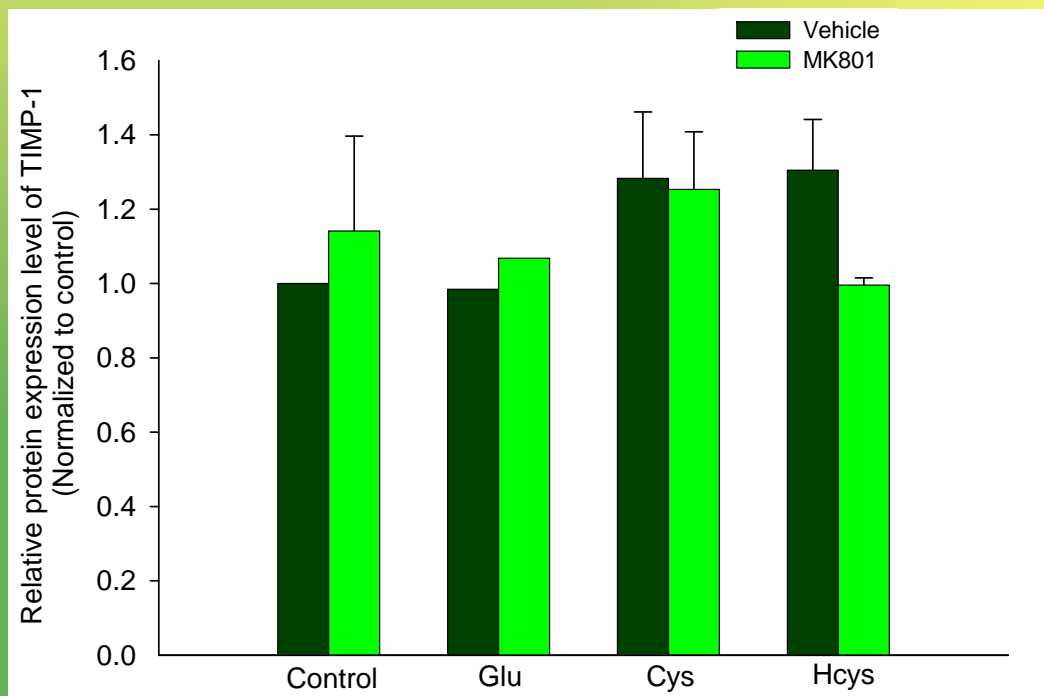
- film exposure and developing

- measure band density

http://www.molecularstation.com/protein/western-blot/

Effect of NMDA-receptor Inhibitor MK801 on the Protein Level of TIMP-1

Summary

- The negative control, Glutathione, does not have a significant effect on either the relative mRNA or protein expression level of TIMP-1.

- Another negative control, Cysteine, does not have an effect on the relative mRNA expression level of TIMP-1, but does cause an increase in the protein expression level. This signifies that Cysteine does not enter the cell through an NDMA-receptor, but through another unknown post-transcription pathway.

- Homocysteine demonstrated significant increases in both the relative mRNA and protein expression levels of TIMP-1.

- NMDA-receptor inhibitor MK801 repeatedly exhibited the tendency to reverse the mRNA and protein expression level increase caused by Homocysteine. This is significant in confirming the pathway of homocysteine within the mesangial cell.

Conclusion

The mRNA detection method of Real-time RT-PCR and the protein detection method of Western Blot Analysis have both confirmed the role of NMDA-receptor as a significant pathway in Homocysteine-induced TIMP-1 expression. NMDA-receptor inhibitor MK801 was able to block and decrease TIMP-1 expression, where in its absence, Homocysteine caused considerably elevated levels of TIMP-1.

Homocysteine-induced TIMP-1 expression is associated with NMDA-receptors mediated homocysteine uptake and stimulation.

Acknowledgement

Dr. Pin-Lan Li
Dr. Ai-Ping Zou

Dr. Fan Yi
Dr. Si Jin
Dr. Fan Zhang
Qi-Zheng Chen
Min Xia
Cindy Thomas
Matt Childrey

Thank you for an unforgettable summer!
- Justine