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# Translocation of H-Ras and its implications in the development of diabetic retinopathy

Renu A. Kowluru *, Mamta Kanwar

*Department of Ophthalmology, Kresge Eye Institute, Wayne State University, USA*

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ABSTRACT

H-Ras, a small molecular weight G-protein, undergoes post-translational modifications enabling its translocation from cytosol to the membrane. Hyperglycemia increases apoptosis of retinal capillary cells via activation of H-Ras, which can be ameliorated by farnesylation inhibitors. Our aim is to investigate the mechanism of retinal H-Ras activation in diabetes. H-Ras and Raf-1 were quantified in the retinal membrane and cytosol fractions obtained from streptozotocin-induced diabetes rats, and the role of post-translation modification was determined by investigating the effect of simvastatin on diabetes-induced alterations. The effect of H-Ras-siRNA on membrane translocation and apoptosis was also determined in bovine retinal endothelial cells (BRECs). Diabetes increased expressions of H-Ras and Raf-1 in the retinal membranes, and simvastatin prevented such translocation. Glucose-exposure of BRECs increased membrane H-Ras expression and H-Ras-siRNA prevented this translocation, and also decreased their apoptosis. Thus, membrane translocation of H-Ras is a plausible mechanism responsible for accelerated apoptosis of retinal capillary cells in diabetes.



## Introduction

Hyperglycemia is considered as the major initiating factor in the development of diabetic retinopathy. Many hyperglycemia-initiated metabolic abnormalities have been implicated in the development of diabetic retinopathy, however, the exact mechanism responsible remains elusive.

H-Ras, a member of small molecular weight GTPases, cycles between a guanosine triphosphate-bound active and a guanosine diphosphate-bound inactive state [1]. It acts as a "molecular switch," converting signals from cell membrane to the nucleus [2]. Post-translational modification of H-Ras activates it by increasing its hydrophobicity and translocating it from cytosol to the membrane [3]. Our studies have shown that H-Ras activation is one of the important steps involved in the apoptosis of retinal endothelial cells and ultimately in the development of diabetic retinopathy, and farnesylation inhibitors inhibit glucose-induced apoptosis of retinal capillary cells [4,5]. Further, H-Ras mediated apoptosis of retinal capillary cells is via its key effector protein, Raf-1, which is predominantly cytosolic [4,5]. Others have shown that the activation of Ras promotes translocation of Raf-1 to the plasma membrane [6,7]. However, how diabetes affects the subcellular translocation of H-Ras in the retina is not explored.

In this study we have investigated the mechanism of H-Ras activation in diabetes. The quantitative measurements of the expression of H-Ras and Raf-1 were made in the retinal membrane of diabetic rats. Simvastatin is shown to block membrane translocation of H-Ras via Ras farnesylation-associated mechanism [8], the effect of administration of simvastatin on diabetes-induced membrane translocation of H-Ras was also investigated. Since retinal capillary cells are the target of histopathology associated with diabetic retinopathy, the effect of H-Ras-siRNA on glucose-induced membrane translocation and apoptosis was determined in isolated retinal endothelial cells.

## Methods

*Rats.* Wistar rats (200–220 g, male) were made diabetic by streptozotocin (55 mg/kg BW), and insulin was administered to diabetic rats to allow slow weight gain while maintaining hyperglycemia (blood glucose 20–25 mM). Age-matched normal rats served as control. A group of diabetic rats received powder diet (Purina 5001) supplemented with 10 mg/kg/day simvastatin (Merck Research Laboratories), and this was initiated soon after establishment of diabetes. Each group had 10–12 rats, and the entire colony of rats (normal, diabetic, and diabetic with simvastatin diet) received fresh powder diet weekly. The rats were euthanized by overdose of pentobarbital at 2 months of diabetes, and the

* Corresponding author. Address: Department of Ophthalmology, Kresge Eye Institute, Wayne State University, 4717 St. Antoine, Detroit, MI 48201, USA. Fax: +1 313 577 8884.
*E-mail address:* rkowluru@med.wayne.edu (R.A. Kowluru).

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retina was removed immediately and used for biochemical measurements. Glycated hemoglobin values (normal = 5–6%, diabetes or diabetes + simvastatin = 11–12%) measured 2–3 days before termination of the experiments using affinity columns (kit 442-B, Sigma Chemicals), showed that simvastatin had no effect on the severity of hyperglycemia. Treatment of the animals conformed to the National Institute of Health Principals of Laboratory Animal Care, the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

*Retinal endothelial cells*. Bovine retinal endothelial cells (BRECs) were grown in medium containing heat inactivated fetal bovine serum and endothelial cell growth supplement [4,5]. Confluent cells from third to sixth passage were incubated in normal (5 mM) or high (20 mM) glucose for 4 days. Control incubations containing 20 mM mannitol always were run simultaneously to rule out the effect of increased osmolarity. All cells received fresh media every 48 h, and each experiment was repeated with three to four different cell preparations.

*Transfection of endothelial cells*. BRECs from third to fifth passage were transfected with H-Ras small interfering RNA (H-Ras-siRNA) using transfection reagents and siRNA duplex from Santa Cruz Biotechnology, CA. The transfection complex was prepared by adding H-Ras-siRNA and siRNA transfection reagent. The mixture was incubated for 30 min at room temperature. The cells were washed and overlayed with the transfection complex, and incubated for 8 h at 37 °C. Parallel incubations were carried out using non-targeting scrambled siRNA, and also the transfection reagent alone (Mock). After the transfection, the media was replaced with the 5 or 20 mM glucose media and the cells were incubated for 4 days. The cells were rinsed with phosphate buffered saline (PBS), trypsinized, and homogenized as given below.

*Cytosol and heavy membrane fractions*. Heavy membranes were prepared by homogenizing retina or cells in 5 mM HEPES buffer (pH 7.4) containing 250 mM mannitol, 70 mM sucrose, 1 mM EGTA, 1 mM DTT, and 10 μg/ml each of leupeptin and aprotinin. The homogenate was centrifuged at 200$g \times 2$ min to remove cell debris, followed by centrifugation at 100,000$g \times 90$ min. The pellet was suspended in homogenization buffer and used as heavy membrane, and the supernatant as cytosol fraction.

*Activation of H-Ras*. The relative abundance of active Ras was quantified using a Raf-1 binding assay kit (Cytoskeleton, Denver, CO). Retinal homogenate was added to Raf-1RBD, and the Raf-RBD/GTP-Ras complex was pulled down by glutathione affinity beads. The amount of activated H-Ras was determined by Western blot using H-Ras-Pan specific antibody [5].

*Western blot analysis*. Proteins (20–30 μg) were subjected to 12% SDS–PAGE and transferred to nitrocellulose membrane. Immunodetection was performed using antibodies against H-Ras and Raf-1 (Santa Cruz Biotechnology). Membranes were re-probed with β-actin to evaluate the lane loading. Band intensities were quantified using Un-Scan-It gel digitizing software (Silk Scientific Inc., UT).

*RNA isolation and quantification*. Total RNA was isolated using TRIZol reagent (Invitrogen, Carlsbad, CA), as routinely used in our laboratory [9]. RNA was converted to single stranded cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, ABI, CA). cDNA was synthesized using the GeneAmp PCR system 9700 thermal cycler (ABI), and quantified spectrophotometrically.

Quantitative RT–PCR was performed in the retina with 50 ng cDNA template using the ABI-7500 sequence detection system, and β-2-microglobulin (B2M) was used as a housekeeping control. Each sample was measured in triplicate. GeneBank Accession Numbers of Taqman custom PCR primers used for H-Ras and B2M are XM_001061671.1 and NM_012512.1, respectively. The standard PCR conditions included 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of extension at 95 °C for 15 s and 1 min at 60 °C. Threshold lines were automatically adjusted to intersect amplification lines in the linear portion of the amplification curves and cycle to threshold ($C_t$) were recorded automatically. Replicated data were normalized with B2M mRNA.

Semi quantitative PCR was performed in BRECs using Gene Amp PCR System 9700 (ABI) for PCR amplification. The forward and reverse primers for H-Ras were: 5′-CCC ACC CTG CCC AAG AG-3′ and 5′-TTG ACG TGG TTG ATA GCA AAC AC-3′, respectively. Equal volumes of reaction mixture from each sample were loaded onto 1.2% agarose gels. The images were digitally captured for analysis of intensity with Un-Scan-It software. Levels of the target gene mRNA was normalized relative to β-actin in the same sample.

*NF-κB activation*. NF-κB activation was determined by ELISA that is based on the principle that only the active form of NF-κB binds to oligonucleotide containing NF-κB consensus site (5′-GGGACTTTCC-3′). The standard concentrations used in the ELISA ranged from 8 to 125 pg/μl [10].

*Nitrite levels*. Nitrite (NO) levels were quantified by Greiss reagent as routinely measured in our laboratory using sodium nitrite as a standard [4,11].

*Cell death*. Cell death was determined by ELISA using Cell Death Detection ELISA$^{PLUS}$ (Roche Diagnostics) [12], and was confirmed by measuring the activity of caspase-3 [13].

*Statistical analysis*. Data are reported as mean ± SD and experimental groups were compared using the non-parametric Kruskal–Wallis test followed by Mann–Whitney test for multiple group comparison. Similar conclusions were also reached by using ANOVA with Fisher or Tukey.

## Results

### *Effect of diabetes on membrane translocation of H-Ras*

The ratio of H-Ras protein expression in membrane to cytosol fractions was increased from 0.5 in normal rat retina to 1.2 in diabetic rat retina (Fig. 1A). In the same diabetic rats the gene expression of H-Ras in the retinal membranes was 1.7-fold higher compared to normal rats (Fig. 1B), and H-Ras was activated by about 40%, as determined by its binding to Raf-1 (Fig. 1C).

As shown in Fig. 1D, the expression of Raf-1 was significantly increased in the membrane fraction in diabetic rats compared to normal rats; the ratio of expression of Raf-1 in membrane to cytosol was 0.7 in normal rat retina and 1.2 in diabetic rat retina.

### *Effect of simvastatin on H-Ras activation*

Administration of simvastatin ameliorated diabetes-induced increases in H-Ras and Raf-1 membrane expression (Fig. 1); the values obtained from simvastatin-treated diabetic rats were significantly different from diabetic rats without simvastatin ($P > 0.5$). This was accompanied by inhibition of retinal H-Ras activation by simvastatin (Fig. 1C).

As shown in Fig. 2A, simvastatin inhibited diabetes-induced activation of retinal NF-κB and decreased NO levels, and the values obtained from simvastatin-treated diabetic rats were lower compared to diabetic rats without any supplementation (Fig. 2B; $P < 0.05$).

### *Membrane expression of H-Ras in retinal endothelial cells*

Membranes obtained from BRECs incubated in high glucose presented significant increases in the protein expression of H-Ras compared to the cells incubated in normal glucose (Fig. 3A). Glucose-induced increase in protein expression of H-Ras was accom-