Potential Contributory Role of H-Ras, a Small G-Protein, in the Development of Retinopathy in Diabetic Rats

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Hyperglycemia is thought to be the underlying factor in the development of diabetic retinopathy, but the mechanisms involved remain partially understood. Diabetes increases oxidative stress, and reactive oxygen species affect the interactions between a small-molecular-weight G-protein, H-Ras, and several of its effector proteins. The purpose of this study was to examine the regulatory role of H-Ras in glucose-induced apoptosis of retinal endothelial cells. The expressions of H-Ras and its effector protein (Raf-1) were compared in the retina obtained from diabetic rats (2–8 months' duration) and age-matched normal rats and in retinal endothelial cells exposed to high-glucose medium. The effect of inhibition of H-Ras function on glucose-induced capillary cell death and the potential involvement of oxidative stress in diabetes-induced activation of H-Ras were also determined. The expressions of H-Ras and Raf-1 were increased in the retina in diabetes, and antioxidant therapy prevented diabetes-induced increased protein and mRNA expressions. The inhibitors of Ras farnesylation inhibited glucose-induced nitric oxides and apoptosis in isolated retinal endothelial cells. Thus, the results suggest that functional activation of H-Ras might be one of the signaling steps involved in glucose-induced capillary cell apoptosis and supports the role of H-Ras in the development of retinopathy in diabetes.
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Diabetic retinopathy is the leading cause of acquired blindness among young adults in developed countries. Hyperglycemia-induced abnormalities in retinal metabolism evidently contribute to the development of the microangiopathy (1–7), but it has been difficult to recognize the abnormalities that are critical for its development. Diabetes increases oxidative stress (3,5,8,9), and reactive oxygen species generated by high glucose are considered causal links between elevated glucose and the other metabolic abnormalities important in the development of diabetic complications (10,11). Oxidative stress is increased in the retina and the retinal capillary cells (both endothelial cells and pericytes) in diabetes (5,9,12–14) and is postulated to play an important role in pericyte dropout seen in diabetic retinopathy. Overexpression of the cell death protease gene in retinal pericytes correlates with the altered gene profile of scavenging enzymes (15). Our studies have shown that long-term administration of multi-antioxidants to diabetic rats inhibits the development of histopathology in the retina and activation of caspase-3 and nuclear factor κB (NF-κB), suggesting that oxidative stress plays an important role in the apoptosis of retinal capillary cells and in the development of diabetic retinopathy (16,17).

GTP-binding proteins of small-molecular-weight (e.g., Ras superfamily of G-proteins) are known to act as biological switches for various cellular processes (18). In mammals, the Ras superfamily includes three highly homologous proteins, namely H-, N-, and K-Ras (19,20). H-Ras is expressed throughout the development, with most predominant expression in the adult brain (18). The Ras superfamily has been implicated in both mitogenic and apoptotic pathways (21–24) and is shown to mediate the upregulation of the vascular endothelial growth factor (VEGF) by triggering the mitogen-activated protein (MAP) kinase pathway. The Ras signaling pathway initiates its interaction with its first effector protein, Raf-1, which is a threonine/serine kinase. The Ras/Raf complex is involved in the signaling steps leading to the induction of intracellular oxidative stress (25), and reactive oxygen species affect the interactions between H-Ras and several of its effector proteins (26,27).

The present study was undertaken to examine the putative regulatory roles of H-Ras in the cascade of events leading to high glucose–induced apoptosis of retinal endothelial cells. This was accomplished by quantitative measurements of the expression of H-Ras and its effector protein (e.g., Raf-1) in the retina in normal rats and diabetic rats and in retinal endothelial cells exposed to high glucose conditions in vitro. The role of H-Ras activation, via its posttranslational prenylation, in the accelerated retinal capillary cell apoptosis was determined. Because long-term administration of antioxidants to diabetic rats inhibits biochemical abnormalities and the development of histopathology in the retina (16,17), we also investigated the potential involvement of oxidative stress in diabetes-induced activation of H-Ras in the retina.

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ELISA, enzyme-linked immunoassay; FTI, farnesylation inhibitor; GGTI, geranyl geranyl transferase inhibitor; LA, α-lipoic acid; MAP, mitogen-activated protein; NAC, *N*-acetyl cysteine; NF-κB, nuclear factor κB; NO, nitric oxide; VEGF, vascular endothelial growth factor.

RESEARCH DESIGN AND METHODS

Rats. Rats (male Wistar, 200–220 g) were randomly assigned to normal or diabetic groups. Diabetes was induced with streptozotocin injection (55 mg/kg body wt i.p.), and insulin was administered to allow slow weight gain while maintaining hyperglycemia (blood glucose levels of 20–25 mmol/l). Soon after establishment of diabetes (3–4 days after administration of streptozotocin), the diabetic rats were divided into four groups. In group 1, the rats received rodent powdered diet (Purina 5001) without any supplementation; group 2 rats received powdered diet supplemented with multi-antioxidants containing 1 g/kg ascorbic acid, 500 mg/kg Trolox, 250 mg/kg DL-α-tocopherol acetate, 200 mg/kg *N*-acetyl cysteine [NAC], 45 mg/kg β-carotene, and selenium, 0.1 mg/kg of diet. The choice and the concentrations of the antioxidants used is based on our previous reports showing that the long-term administration of this multi-antioxidant therapy inhibits diabetes-induced activation of retinal caspase-3 and NF-κB and the development of retinopathy in rats (16,17). Diabetic rats in group 3 received the diet supplemented with α-lipoic acid (LA) (400 mg/kg), and in group 4, the rats received diet supplemented with NAC (2 g/kg). Each group had 10 or more rats. The entire colony of rats (normal, diabetic, and diabetic with supplemented diets) received fresh diet weekly. The rats were weighed two times a week, and their food consumption was measured once every week to calculate the amount of antioxidants consumed. GHb was measured at 2 months of diabetes and every 3 months thereafter, using affinity columns (kit 442-B; Sigma). Rats (diabetic and age-matched normal control) were killed at 2 and 8 months of diabetes, and the retina was immediately removed. Treatment of 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, and the institutional guidelines.

Capillary cells. Retinal endothelial cells were prepared from bovine eyes by the method routinely used by our laboratory (16,28,29). Endothelial cells were grown to 80% confluence in Petri dishes coated with 0.1% gelatin in Dulbecco's modified Eagle's medium containing heparin, 10% fetal calf serum (heat inactivated), 10% Nu-serum, endothelial growth supplement (25 μg/ml), and antibiotic/antimycotic in an environment of 95% O_2 and 5% CO_2. Confluent cells from the fourth to sixth passage were split and incubated in normal (5 mmol/l) or high (20 mmol/l) glucose for 1–5 days in the presence or absence of either farnesylation inhibitors (FTIs): 25 μmol/l FTI-277, 20 μmol/l geranyl geranyl transferase inhibitors (GGTIs), 10 μmol/l manumycin, 20 μg/ml damancanthol, or 25 μmol/l 3-allyl FTI or 250 μmol/l of the antioxidants NAC or LA (16,28). Control incubations containing 20 mmol/l mannitol were always run simultaneously to rule out the effect of increased osmolarity. Each experiment was repeated with at least three separate cell preparations.

Expression of H-Ras and Raf-1. Forty-μg of protein was separated on a 10–15% denaturing polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked and that was followed by incubation of the membranes with a polyclonal antibody against H-Ras, K-Ras, or Raf-1 (Santa Cruz Biotechnology). The membranes were washed, incubated with anti-rabbit IgG horseradish peroxidase–conjugated antibody (Amersham Biosciences), and developed using an ECL-Plus Western blotting detection kit (Amersham Biosciences). Kaleidoscope prestained molecular weight markers (Bio-Rad) were run simultaneously on each gel. To ensure equal loading among the lanes, the expression of housekeeping protein β-actin was also determined. After blotting the membrane for the desired proteins, the membranes were incubated with stripping buffer (62.5 mmol/l Tris-HCl, pH 6.8, 100 mmol/l mercaptoethanol, 2% sodium dodecyl sulfate) at 50°C for 30 min, washed, and incubated with antibody against β-actin (monoclonal, anti-mouse; Sigma). IgG horseradish peroxidase–conjugated anti-mouse antibody was used as the secondary antibody, and the membranes were developed using an ECL-Plus Western blotting detection kit. Each sample was analyzed in duplicate.

mRNA levels. To determine whether diabetes has any effect on the mRNA levels of H-Ras, mRNA of H-Ras was quantified in the retina by real-time PCR. Total RNA was extracted using Trizol (Invitrogen), the samples were centrifuged (12,000*g* × 15 min), and RNA was isolated from the aqueous layer and precipitated using isopropyl alcohol. RNA (3 μg) was used for cDNA synthesis according to the methods described previously (30). Briefly, RNA was mixed with oligo-d(T) primers and incubated for 10 min at 70°C. The samples were quenched on ice, and reverse transcription was carried out by adding dNTPs and Moloney murine leukemia virus–reverse transcriptase.

Real-time RT-PCRs were carried out in a LightCycler (Roche Diagnostics) using DNA FastStart SYBR Green I Master Mix (Roche Diagnostics). The reaction mixture consisted of 2 μl FastStart DNA SYBR Green I master mix, 1 μl of each forward and reverse 10 μmol/l primers (forward, 5′-cagagctcctg gtttggcag-3′; reverse, 5′-tctatagagggatcatactc-3′), 1.6 μl of 25 mmol/l $MgCl_2$, 13.4 μl H_2O, and 1 μl cDNA. During PCRs, an additional signal acquisition step was added after the elongation phase to allow for signal acquisition specifically from the target sequence. The mRNA levels were quantified by the standard curve method using serially diluted standard template. The cycle number (start of exponential phase, as determined by the LightCycler software) was used to compute the relative levels of mRNAs from the standard curve. mRNA for H-Ras was normalized to 18S rRNA to account for any differences in reverse transcription efficiency and amount of template in the reaction mixture.

Confocal microscopy. To investigate possible localization of H-Ras in the retinal endothelial cells and the effect of high glucose, the cells incubated in 5 or 20 mmol/l glucose for 3 days were fixed with methanol:acetone (1:1) and blocked in PBS containing normal goat serum and BSA. The cells were stained using polyclonal rabbit antibody against H-Ras (Santa Cruz Technology), washed with PBS, and incubated with secondary antibody (anti-rabbit IgG specific)-fluorescein isothiocyanate (Sigma). The slides were mounted in Vectashield (Vector Laboratories), and microscopy was performed using a Zeiss LSM 310 inverted laser scan microscope equipped with fluorescein filter. The controls were run parallel by substituting the blocking solution for H-Ras antibody. Average pixel intensities within each cell were quantified using the Image J software.

Nitrite levels. Nitrite production was measured using a Greiss reagent (31,32). The absorbance was measured at 540 nm, and the nitrite concentration was calculated from a sodium nitrite standard curve. These measurements were not influenced by the inclusion of antioxidants, FTIs, or GGTIs.

Cell death. Cell death was determined by enzyme-linked immunosorbent assay (ELISA) and by DNA laddering techniques using Cell Death Detection ELISA^{PLUS} and Apoptotic DNA ladder kits (Roche Diagnostics) (29) and was confirmed by measuring the enzyme activity of caspase-3 (16).

Monoclonal antibodies directed against DNA and histones, respectively, were used to quantify the relative amounts of mono- and oligonucleosomes generated from apoptotic cells. The cytoplasmic fraction of the cells was transferred onto a streptavidin-coated microtiter plate and incubated for 2 h at room temperature with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled anti-histone. The plate was washed thoroughly and incubated with the ABTS (Roche Diagnostics), and absorbance was measured at 405 nm. After separation of the cytoplasmic fraction, the nuclear pellet was suspended in 50 mmol/l sodium phosphate buffer (pH 7.5) containing 2 mmol/l NaCl and 0.05 mmol/l Na_2HPO_4 (pH 7.5) and sonicated. DNA was measured in this fraction, and apoptosis was normalized to the amount of DNA (29).

DNA fragmentation was detected using the Apoptotic DNA Ladder Kit. Briefly, the washed cells were resuspended in PBS and incubated for 10 min with an equal volume of buffer containing 10 mmol/l Tris-HCl (pH 7.4), 6 mol/l guanidine-HCl, 10 mmol/l urea and EDTA, and 0.2% Triton X-100. The samples were passed through the glass fiber fleece by centrifugation, and nucleic acid bound to the glass fibers was eluted. The DNA was applied on 1.5% agrose gel, and the bands were then visualized by ethidium bromide staining and photographed.

Caspase-3. Activation of caspase-3 in the retina was determined by measuring the cleavage of a fluoregenic substrate specific for caspase-3, *N*-acetyl-As-Glu-1-Val-asp-7-amino-4 trifluoromethyl coumarin (DEVD-AFC; BIOMOL Research Laboratories). The fluorescence signal emitted by cleavage of the substrate was quantified at excitation and emission wavelengths of 400 and 505 nm, respectively (16). Each sample was measured in duplicate.

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

RESULTS

Effect of diabetes on H-Ras in the retina. As shown in Fig. 1, the protein expression of H-Ras was increased by over twofold in the retina of rats diabetic for 2 months compared with age-matched normal rats ($P < 0.05$). A similar increment in H-Ras expression was also observed when the duration of diabetes was increased to 8 months (a duration when capillary cell apoptosis can be observed in the retina [33]). Despite differences in the expression of H-Ras, the content of the intrinsic protein β-actin did not vary among different lanes. To rule out the possibility that the activation of H-Ras is a systemic effect of diabetes, its expression was also determined in the cerebral cortex, the tissue that is resistant to diabetes-induced oxidative stress and complications (12). The results in Fig. 1*B* show that

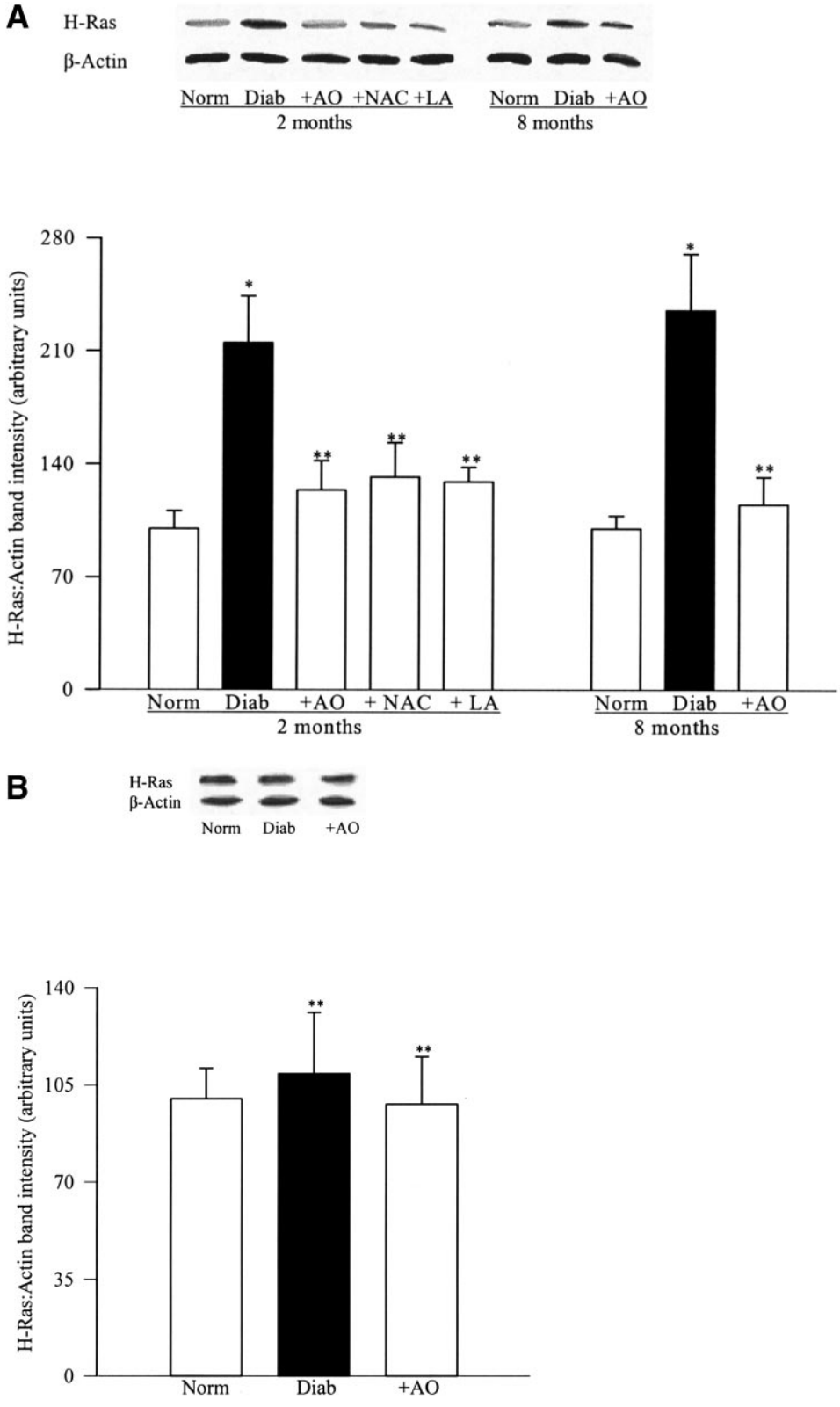


FIG. 1. Effect of diabetes on the H-Ras in rat (*A*) retina (*B*) cerebral cortex: the expression of H-Ras was determined using a polyclonal antibody against H-Ras in the retina and cerebral cortex obtained from rats diabetic for 2 or 8 months and their age-matched normal rats. The band intensities for H-Ras were adjusted to the expression of β-actin in each lane. The histogram represents the absorbance of H-Ras: β-actin bands in each lane. The samples from each rat were analyzed in duplicate in two separate experiments. AO, antioxidant. *$P < 0.05$ and **$P > 0.05$ compared with the age-matched normal controls. *A*: The Western blots presented for retina are representative of five rats in each of the two normal (Norm) control and diabetes (Diab) groups (2- and 8-month durations) and four rats each in the diabetes +NAC, +LA, or +AO groups. *B*: The cerebral cortex was obtained from the same normal, diabetes, and diabetes +AO rats (2 months' duration) as the retina. The Western blots are representative of four rats each in the normal and diabetes +AO groups, and five rats in the diabetes group.

diabetes has no effect on the expression of H-Ras in the cerebral cortex.

To determine whether a diabetes-induced increase in retinal H-Ras is via an increase in its mRNA, the latter was determined by real-time RT-PCR. mRNA levels of H-Ras were increased by threefold in the retina at 2 months of diabetes compared with that obtained from age-matched normal rats ($P < 0.05$, Fig. 2). When the duration of diabetes in rats was extended to 8 months, no additional increase in the abundance of H-Ras mRNA was observed (data not shown).

Administration of multi-antioxidants that are shown to inhibit the development of retinopathy and activation of caspase-3 and NF-κB in diabetic rats (9,16,17) for the

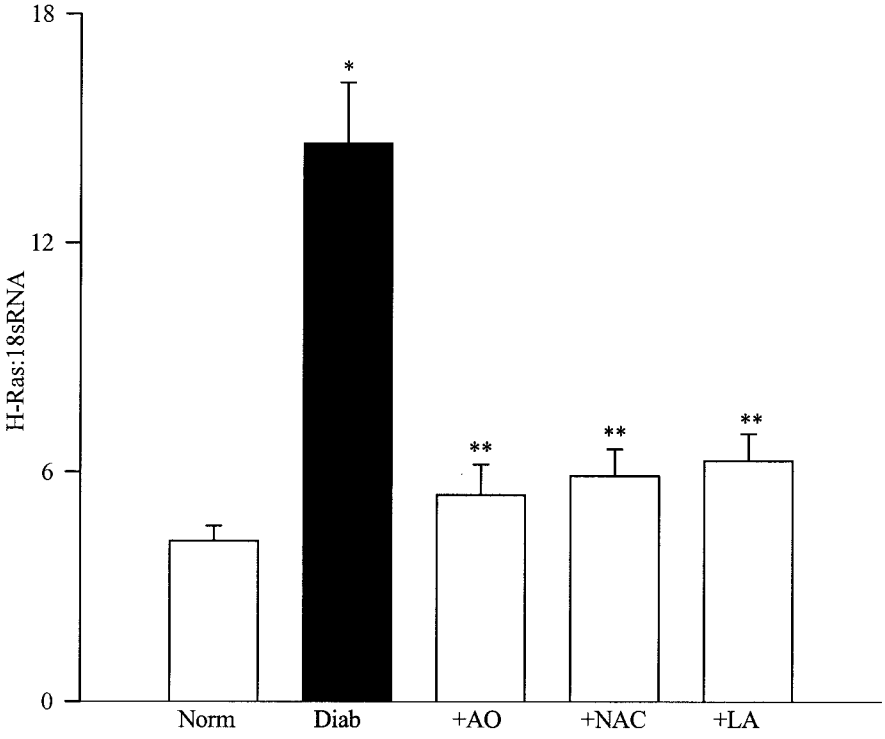


FIG. 2. Effect of diabetes on H-Ras mRNA content in the retina: mRNA content was determined in the retina of rats diabetic for 2 months and in normal control rats by real-time RT-PCR using 5′-cagagctcctggtttggcag-3′ and 5′-tctatagagggatcatactc-3′ as forward and reverse primers, respectively. The mRNA levels for H-Ras were normalized to 18SrRNA. The results are presented as means ± SD of five rats each in the normal, diabetes, and diabetes +NAC groups and four rats each in the diabetes +AO and diabetes +LA groups. Retina from each rat was analyzed at least in duplicate. *$P < 0.05$ and **$P > 0.05$ compared with normal controls. AO, antioxidant.

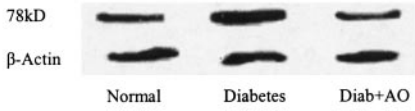
entire duration of diabetes (2–8 months) significantly inhibited increases in the expressions (both protein and mRNA) of H-Ras (Figs. 1 and 2). Similarly, when the diabetic rats were fed diets supplemented with either NAC or LA (2 months' duration, the only duration studied with NAC and LA), increases in the expression of protein and mRNA for H-Ras were markedly attenuated.

In the same retinal samples, the expression of the effector protein for Ras, Raf-1, was also increased significantly in diabetes, and antioxidants inhibited such increases (Fig. 3).

Effect of antioxidants on the severity of hyperglycemia in diabetic rats. Administration of antioxidants (NAC or LA) had no effect on the body weight and 24-h urine excretion (>90 ml/24 h in the animals in all of the four diabetic groups compared with <20 ml in normal controls). GHb values from diabetic rats were similarly elevated (11–13%) compared with levels in normal control rats (4–5%).

Localization of H-Ras in endothelial cells and effect of high glucose on H-Ras expression. Our results using confocal microscopy revealed localization of H-Ras in the retinal endothelial cells (Fig. 4). When the cells were incubated in high glucose for 3 days, fluorescein intensity for H-Ras (the average pixel intensities within each cell, quantified using Image-J program) was increased by 25% compared with the cells incubated in 5 mmol/l glucose ($P < 0.05$ compared with normal, Fig. 4*B*).

Incubation of retinal endothelial cells in 20 mmol/l glucose for 3 days resulted in about an 80% increase in the protein expression of H-Ras compared with the cells incubated in 5 mmol/l glucose ($P < 0.05$ compared with 5 mmol/l glucose, Fig. 5). When the duration of incubation with 20 mmol/l glucose was increased to 10 days, the expression of H-Ras did not show any additional increase (data not shown). Compatible with our in vivo data



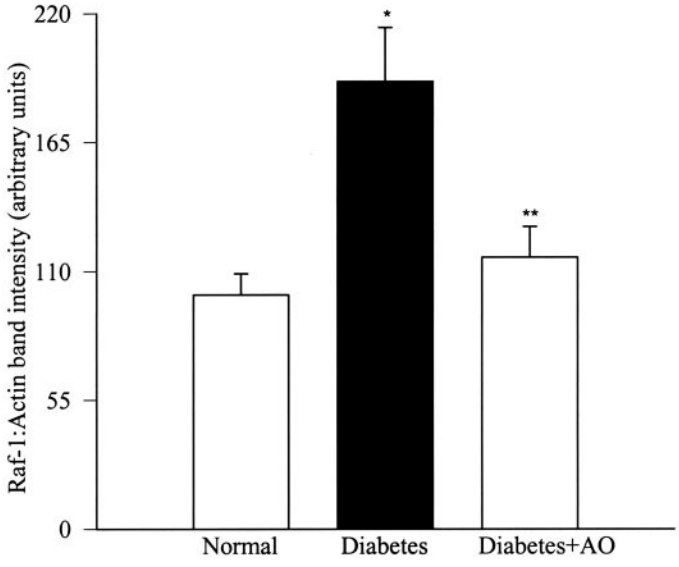


FIG. 3. Raf-1 expression in the retina. Raf-1 content in the retina obtained from the rats diabetic for 2 months receiving normal or antioxidant (AO) diet was determined using polyclonal antibody against Raf-1. The Western blot represents the results obtained from at least four rats in each group, with each retina analyzed in duplicate. *$P < 0.05$ and **$P > 0.05$ compared with normal control.

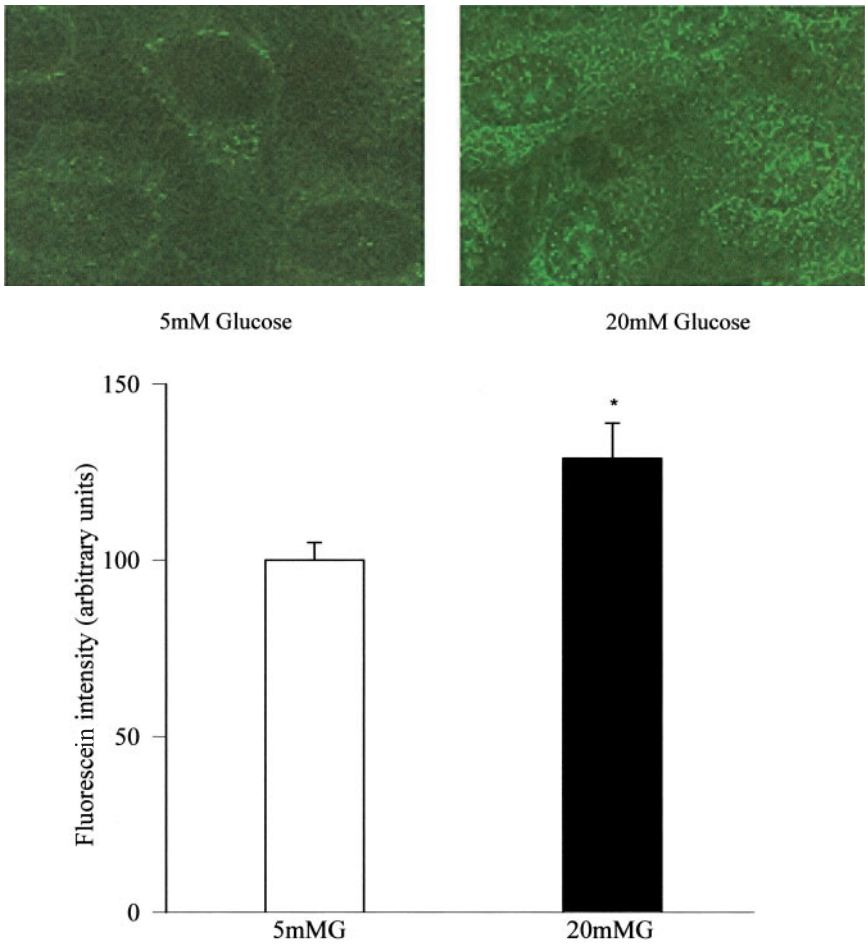


FIG. 4. Confocal images of H-Ras. Retinal endothelial cells exposed to 5 mmol/l glucose (left) and 20 mmol/l glucose (right) for 3 days were fixed with methanol:acetone, blocked, and stained using polyclonal rabbit H-Ras antibody (Santa Cruz Technology). Anti-rabbit IgG-specific FITC was used as the secondary antibody. The microscopy was performed using a Zeiss LSM 310 inverted laser scan microscope equipped with fluorescein filter. The experiment was repeated three times using different cell preparations. Average pixel intensities within each cell were quantified using Image-J = program. The histogram represents the means ± SD pixel intensities obtained from at least 15 cells in each incubation condition.

represented above, inclusions of NAC or LA, the antioxidants that are shown to inhibit glucose-induced oxidative stress and caspase-3 activation (16), inhibited a glucose-induced increase in H-Ras content. Addition of 20 mmol/l mannitol, instead of glucose, failed to increase H-Ras expression, suggesting that glucose-induced elevation in H-Ras was not due to an increase in the osmolarity (data not shown).

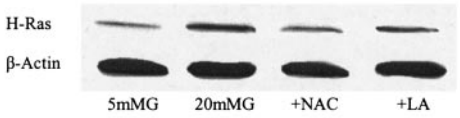
Inhibitors of Ras farnesylation and glucose-induced nitric oxide and cell death. The structurally dissimilar inhibitors of protein farnesylation, FTI-277, manumycin, damancanthol, and 3-allylFTI significantly inhibited a glucose-induced increase in nitric oxide (NO) ($P < 0.05$ compared with 20 mmol/l glucose, Fig. 6). However, these inhibitors had no significant effect on the basal NO levels obtained from 5 mmol/l glucose conditions. To confirm that farnesylation of Ras, rather than geranyl geranylation of other small G-proteins, is involved in this signaling cascade, GGTI (a selective inhibitor of geranyl geranylation) was included in the incubation medium, and our results show that GGTI had no effect on glucose-induced NO.

The incubation of endothelial cells in 20 mmol/l glucose medium for 5 days markedly increased (70%) their apoptosis compared with that observed from the cells incubated in 5 mmol/l glucose medium ($P < 0.05$), and addition of manumycin or FTI-277 inhibited such apoptosis (Fig. 7*A*). Similarly manumycin and FTI-277 also inhibited glucose-induced DNA-laddering in the endothelial cells (data not shown).

To further confirm that H-Ras activation plays a crucial role in the glucose-induced accelerated endothelial cell apoptosis, effect of FTI-277 and manumycin were determined on the enzyme activity of caspase-3. As shown in Fig. 7*B*, the activity of caspase-3 was increased by 60% in the endothelial cells incubated in high glucose for 5 days, and inclusion of manumycin during the incubation inhibited glucose-induced activation of the enzyme. Similar beneficial effects were obtained when manumycin was replaced by FTI-277.

DISCUSSION

The results presented here show for the first time that expression of H-Ras as well as its effector protein Raf-1 are increased in the retina in diabetes and strongly suggest a role of H-Ras in the glucose-induced apoptotic demise of the retinal capillary cells. We also demonstrated that post-translational prenylation of Ras is critical for glucose-mediated effects, because inhibitors of Ras farnesylation (and hence its function) inhibited glucose-induced NO and subsequent apoptosis of retinal capillary cells. Together, these data suggest that functional activation of H-Ras is one of the signaling steps involved in glucose-induced capillary cell apoptosis that has been reported in diabetes (33,34). Further, the same antioxidant therapy that inhibits the development of retinal pathology and activation of apoptosis execution enzyme in diabetic rats (16) prevents diabetes-induced increased protein and mRNA expression



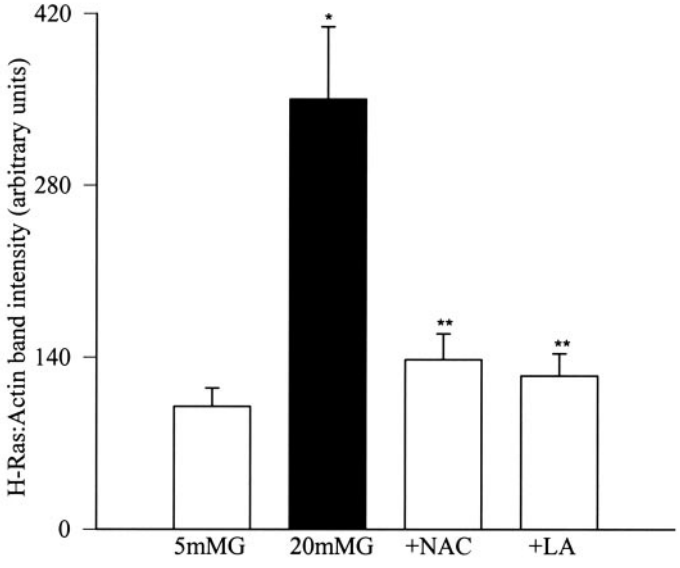


FIG. 5. H-Ras activation in the retinal endothelial cells incubated in high-glucose medium. H-Ras expression was measured in endothelial cells incubated in 5 and 20 mmol/l glucose medium for 3 days. Each measurement was performed in duplicate using three (or more) cell preparations. The values obtained at 5 mmol/l glucose are considered 100%. 5mMG, 5 mmol/l glucose; 20mMG, 20 mmol/l glucose; +LA, 20 mmol/l glucose + 0.25 mmol/l LA; +NAC, 20 mmol/l glucose + 0.25 mmol/l NAC. *$P < 0.05$ and **$P > 0.05$ compared with 5 mmol/l glucose.

of H-Ras in the retina. The results are compatible with recent reports of the involvement of H-Ras in cytokine-induced metabolic dysfunction and apoptosis of the islet β-cells (32) that have demonstrated the importance of H-Ras activation in the induction of inducible NO synthase and subsequent NO release and caspase-3 activation. Together, these data suggest that functional activation of H-Ras is one of the signaling steps involved in glucose-induced retinal capillary cell apoptosis that has been reported in diabetes (33,34).

Increased oxidative stress is experienced by the retina and its isolated capillary cells in hyperglycemia, and increased oxidative stress is postulated to play an important role in the development of diabetic complications. One of the key regulators of the signaling cascade triggered by oxidative stress is the family of small-molecular-weight G-proteins (25). The targets for oxidizing agents are located upstream as well as downstream of H-Ras, and reactive oxygen species can affect the interactions between Ras and several of its ligands (26,27). In endothelial cells from the human umbilical vein, increases in intracellular reactive oxygen species trigger H-Ras activation, and activated H-Ras could further stimulate the levels of reactive oxygen species by activating NADPH oxidase (25,35). Further, reactive oxygen species produced by advanced glycation end products are shown to stimulate the Ras/Raf/MEK signaling cascade (36), and NF-κB is an important player in the activation of mitogen-activated protein (MAP) kinase (37). We have shown that the activation of retinal NF-κB in diabetes is an early event in the development of retinopathy (17), and the data presented here show that the antioxidants that inhibits the activation of NF-κB and the development of retinopathy in diabetic rats inhibits activation of both H-Ras and Raf-1 in the retina. In addition, our data clearly illustrate that the antioxidants (NAC and LA) that are reported to inhibit glucose-induced retinal capillary cell apoptosis (29) also inhibit H-Ras activation in the retinal endothelial cells, suggesting that increased activation of H-Ras in diabetes is associated

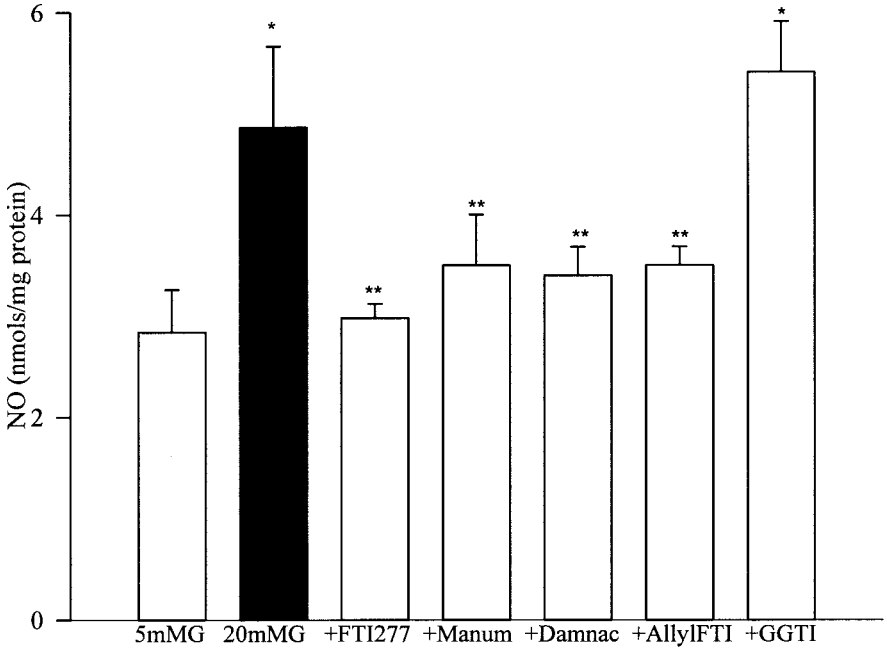


FIG. 6. Effect of inhibitors of H-Ras function on glucose-induced NO in retinal endothelial cells. NO content was measured in the cells incubated in 5 or 20 mmol/l glucose for 3 days in the presence or absence of the inhibitors. Each measurement was performed in duplicate. 5mMG, 5 mmol/l glucose; 20 mMG, 20 mmol/l glucose; +Allyl FTI, 20 mmol/l glucose + 25 μmol/l 3-allyl FTI; +Damnac, 20 mmol/l glucose + 20 μg/ml damancanthol; +GGTI, 20 mmol/l glucose + 20 μmol/l GGTI; +FTI-277, 20 mmol/l glucose + 25 μmol/l FTI-277; +Manum, 20 mmol/l glucose + 10 μmol/l manumycin. *$P < 0.05$ and **$P > 0.05$ compared with 5 mmol/l glucose.

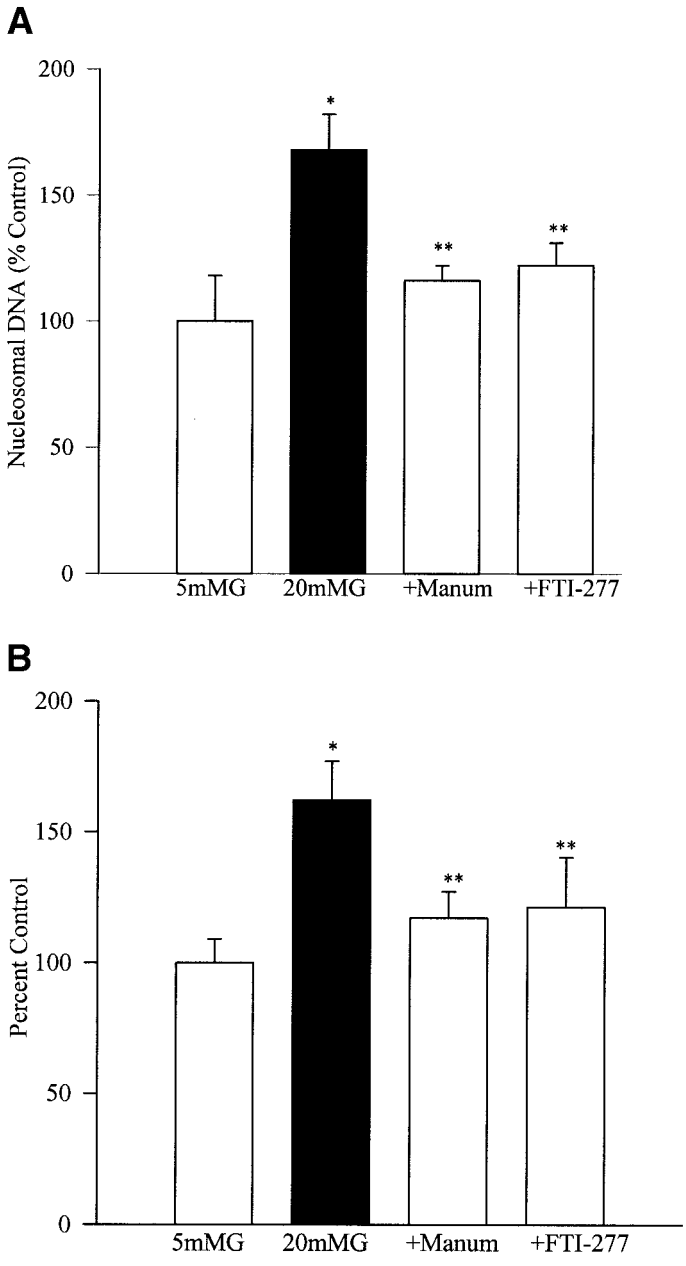


FIG. 7. Effect of FTIs on capillary cell death. *A*: Apoptosis was measured by performing ELISA for cytoplasmic histone-associated DNA fragments using an assay kit from Roche Diagnostics. The graph shows the values obtained from the cells incubated with glucose for 5 days, and these values were adjusted to the total DNA. *B*: The effect of FTIs on the activation of apoptosis execution enzyme caspase-3 was determined in the cells using the fluoregenic substrate *N*-acetyl-As-Glu-1-Val-asp-7-amino-4 trifluoromethyl coumarin (DEVD-AFC). The fluorescence signal emitted was quantified at excitation and emission wavelengths of 400 and 505 nm, respectively. Each experiment was repeated with at least three different cell preparations, and measurements were done in duplicate. The values obtained with 5 mmol/l glucose were considered 100%. 5mMG, 5 mmol/l glucose; 20mMG, 20 mmol/l glucose; +FTI-277, 20 mmol/l glucose + 25 μmol/l FTI-277; +Manum, 20 mmol/l glucose + 10 μmol/l manumycin. *$P < 0.05$ and **$P > 0.05$ compared with 5 mmol/l glucose.

with retinal capillary cell apoptosis. However, we cannot rule out the possible contribution of nonenzymatic glycation in the activation of H-Ras and Raf-1, since nonenzymatic glycation of small G-proteins in diabetes is not well documented. Our in vivo results show that antioxidants inhibit diabetes-induced activation of retinal H-Ras in the retina despite similar glycated hemoglobin levels in the diabetic control group and the diabetic group receiving antioxidants, suggesting that nonenzymatic glycation may not be an important player in the activation of H-Ras in diabetes.

Ras is highly expressed throughout the development (18), and our data show that the protein expression and mRNA levels of H-Ras are increased in the retina in diabetes and in isolated endothelial cells incubated in high glucose. In contrast, 2 months of diabetes has no effect on the protein expression of H-Ras in the cerebral cortex obtained from the same animals, suggesting that the increase in H-Ras seen in the retina is not due to the systemic effects of diabetes. Further, positive staining for H-Ras is seen in the retinal endothelial cells, and the staining is increased when the cells are incubated in high glucose conditions compared with the cells incubated in normal glucose conditions, suggesting that capillary cells in the retina represent one of the target sites for H-Ras–mediated effects.

Our data indicate that the expression of Raf-1 is also increased in the retina obtained from diabetic rats that can be inhibited by the antioxidants. Raf-1 is a key effector protein of Ras function, and in its inactive state, it is predominantly cytosolic. Activation of Ras promotes translocation of Raf-1 to the plasma membrane, leading to phosphorylation of Raf-1, and that results in phosphorylation of MAP/extracellular signal–related kinases (38). The Ras superfamily has been implicated in the apoptotic pathways (21,22,39) and is shown to mediate the upregulation of VEGF via triggering the MAP kinase pathway. The MAP kinase pathway is considered as one of the major components of the signal transduction mediated by Ras. Gene array studies have shown that the mRNA expression of MAP kinase increases in the retina as early as 3 days after induction of diabetes in Long-Evans rats (40), and MAP/extracellular signal–related kinase activation is observed also in retina obtained from diabetic rats (41).

Ras proteins exist either in an inactive GDP-bound form or in an active GTP-bound conformation. Guanine nucleotide exchange factors promote dissociation of GDP from the inactive Ras-GDP complex, allowing Ras proteins to bind GTP, and the activation requires posttranslational modification of Ras proteins (farnesylation). The process of farnesylation of Ras is catalyzed by farnesyltransferase (42). The posttranslational modifications of Ras make it more hydrophobic, facilitating in their translocation from the cytosol to the membrane (42). In human umbilical endothelial cells, insulin is shown to activate farnesyltransferase, and this activation is likely to augment the vascular actions of VEGF (43). In corneal epithelial cells, Ras is proposed to be involved in the signal transduction pathways induced by the glial cell–derived neurotrophic factor, suggesting that Ras is involved in corneal wound healing (44). In addition, Ras and Raf-1 are considered to be the key components in signaling for retinal pigment epithelial cell proliferation (45). The data are presented here showing that, in diabetes, retinal H-Ras and Raf-1 expressions are increased and can be inhibited by antioxidants.

FTIs are shown to eliminate the sensor and the main inducer of reactive oxygen species, leading to the escape of the cell from oxidative stress–induced apoptosis (25).

Recent studies, including our own, in isolated β-cells using inhibitors of posttranslational modifications of Ras have suggested that H-Ras activation is essential for NO release (32,46,47). Here we present data showing that the inhibitors of protein farnesylation that are structurally dissimilar significantly inhibit glucose-induced increase in NO and cell death in the retinal endothelial cells. This strongly suggests that H-Ras activation plays a crucial role in endothelial cell apoptosis seen in hyperglycemia. These FTIs also inhibited glucose-induced activation of caspase-3, the enzyme that is a key target involved in the high glucose-induced apoptosis in human endothelial cells. In support of our results, inhibition of protein farnesylation is reported to prevent advanced glycation end product–induced signaling pathways in endothelial cells and inhibit the corneal opacity and neovascularization induced by an inflammatory stimulus (48,49). We need to acknowledge that our results do not provide any clear evidence as to how complete the pharmacological inhibition of Ras farnesylation was under experimental conditions. However, based on extant data in other cells and the K_i values of these inhibitors, it is likely that, under our current assay conditions, the farnesylation of the biologically active pool of H-Ras should have been completely inhibited.

Although the relative contributory role of H-Ras in the pathogenesis of diabetic complications remains unclear, recent evidence tends to support a role for protein prenylation in this signaling cascade. For example, pravastatin, an inhibitor of HMG-CoA reductase and hence the biosynthesis of isoprenoids required for Ras prenylation, inhibits the development of diabetic nephropathy and inhibits glucose-induced Ras and MAP kinase activities in the mesangial cells cultured in high glucose, suggesting the role of Ras-mediated pathways in the development of nephropathy (50). Our studies suggest the role of H-Ras in the development of retinopathy in diabetes. Taken together, our data provide a basis for the future investigations on the potential therapeutic implications in the use of Ras farnesylation inhibitors to inhibit the development of retinopathy in diabetes.

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