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PUBLISHER'S NOTE

Publisher's Note – Relating to the retraction of: Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration. Azza B. El-Remessy, Manuela Bartoli, Danial H. Platt, David Fulton, Ruth B. Caldwell. J. Cell Sci. doi:10.1242/jcs.195966

Michael Way
Editor-in-Chief, Journal of Cell Science (jcs@biologists.com)

This Publisher's Note concerns the retraction notice (*J. Cell Sci.* 2016 **129**, 3203 doi: 10.1242/jcs.195966) relating to the article 'Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration' by Azza B. El-Remessy, Manuela Bartoli, Danial H. Platt, David Fulton, Ruth B. Caldwell. *J. Cell Sci.* 2005 **118**, 243-252 (doi: 10.1242/jcs.01612).

Journal of Cell Science would like to clarify the following points:

- Although involved in the investigation, the Charlie Norwood VA Medical Center did not exercise jurisdiction over the conclusions of the investigation of the paper named above.
- UGA was presented with the professional opinions of select independent experts in the same field of research as Dr El-Remessy that disputed the findings of research misconduct, but University of Georgia stood by their initial decision to recommend retraction of the paper named above.
- Dr El-Remessy does not agree with the findings of the investigation.

Journal of Cell Science

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RETRACTION

Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration

Azza B. El-Remessy, Manuela Bartoli, Danial H. Platt, David Fulton and Ruth B. Caldwell

Retraction of: *J. Cell Sci.* **118**, 243-252.

Journal of Cell Science was contacted by a reader who alerted us to potential band duplication and manipulation in Fig. 3B, Fig. 4A,B and Fig. 5B in this paper. We contacted the corresponding author, Dr Azza El-Remessy, but she was unable to provide us with a satisfactory explanation. We therefore referred the matter to the institutes at which the research was performed by Dr El-Remessy, University of Georgia and Augusta University (formerly Georgia Regents University), for investigation.

A joint investigatory committee between the two institutes, as well as the Charlie Norwood Veterans Affairs Medical Center, where Dr El-Remessy has additional appointments, concluded that Dr El-Remessy committed research misconduct by falsification or fabrication in the above-named article. Specifically, evidence of direct and mirrored duplication or band substitution was detected in Fig. 3B, Fig. 4A,B and Fig. 5B.

These findings undermine the integrity of the presented findings, and Journal of Cell Science is therefore following the recommendation of the committee to retract this article.

Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration

Azza B. El-Remessy[1,2,*], Manuela Bartoli[1,3], Danial H. Platt[1], David Fulton[1,2] and Ruth B. Caldwell[1,4,5]

[1]Vascular Biology Center, [2]Department of Pharmacology, [3]Department of Pathology, [4]Department of Cellular Biology and Anatomy and [5]Department of Ophthalmology, Medical College of Georgia, Augusta, GA 30912, USA

*Author for correspondence (e-mail: aelremessy@mcg.edu)

Accepted 20 October 2004
Journal of Cell Science 118, 243-252 Published by The Company of Biologists 2005
doi:10.1242/jcs.01612

Summary

In diabetic retinopathy, endothelial cell apoptosis is paradoxically increased despite upregulation of the potent pro-survival factor VEGF. We tested the hypothesis that elevated glucose levels disrupt VEGF's pro-survival function via peroxynitrite-mediated alteration of the Akt-1/p38 MAP kinase signaling pathway by studies of retinal endothelial cells in vitro. High glucose or exogenous peroxynitrite caused significant increases in apoptosis in the presence or absence of VEGF. Treatment with a peroxynitrite decomposition catalyst blocked these effects, implying a causal role of peroxynitrite. Peroxynitrite or high glucose treatment also increased phosphorylation of p38 MAP kinase, whereas phosphorylation of Akt-1 was significantly decreased in basal or VEGF-stimulated conditions. High glucose- or peroxynitrite-treated cells also showed significant increases in tyrosine nitration of p85 subunit of PI 3-kinase that blocked PI 3-kinase and Akt-1 kinase activity. Inhibiting peroxynitrite formation or blocking tyrosine nitration of p85 restored the activity of PI 3-kinase and Akt-1 kinase, blocked phosphorylation of p38 MAP kinase and normalized pro-survival function. Transfecting the cells with constitutively active Akt-1 or inhibiting activity of p38 MAP kinase completely masked the pro-apoptotic effects of high glucose and exogenous peroxynitrite, suggesting an interaction between the Akt-1 and p38 MAP kinase pathways. In conclusion, high glucose treatment blocks the pro-survival effect of VEGF and causes accelerated endothelial cell apoptosis via the action of peroxynitrite in causing tyrosine nitration of PI 3-kinase, inhibiting activity of Akt-1 kinase and increasing the activity of p38 MAP kinase.

Key words: Peroxynitrite, High glucose, Apoptosis, Endothelial cells, p38 MAP kinase, Tyrosine nitration, Akt-1

Introduction

The initial stages of diabetic retinopathy are believed to lead to sight-threatening ischemic and proliferative retinopathy when a critical number of retinal capillaries become non-perfused. It has been reported that retinal endothelial cells in diabetic patients and animals undergo accelerated death by a process consistent with apoptosis (Mizutani et al., 1996). Vascular endothelial growth factor (VEGF), an angiogenic cytokine secreted by a variety of cells, is a potent survival factor for endothelial cells in vivo and in vitro (Gerber et al., 1998). Paradoxically, even though levels of VEGF and VEGF receptor (VEGFR2) are substantially increased in diabetic retinas (Aiello et al., 1994; Duh and Aiello, 1999; El-Remessy et al., 2003b; Gardner et al., 2002; Joussen et al., 2002; Obrosova et al., 2001; Obrosova et al., 2003) and retinal endothelial cell death is also increased.

Vascular endothelial cells are an important target of hyperglycemic damage (Nishikawa et al., 2000). It has been reported that high glucose is associated with formation of reactive oxygen species and that glucose-induced oxidative stress contributes to the development of hyperglycemia-induced vascular injury (Ceriello et al., 2001; Ceriello et al., 2002; Cosentino et al., 1997; Graier et al., 1999). We have shown increased formation of nitric oxide, superoxide anion and their combination product peroxynitrite in retinal endothelial cells maintained in high glucose (El-Remessy et al., 2003a).

Peroxynitrite is a highly reactive oxidant that mediates a variety of biological processes including inhibition of key metabolic enzymes, lipid peroxidation, nitration of the protein tyrosine residues and reduction of cellular antioxidant defenses by oxidation of thiol pools (Misko et al., 1998; Salgo et al., 1995). Recently, it has been shown that increased levels of nitrotyrosine correlate with cell death in pancreas (Suarez-Pinzon et al., 1997) and in a model for oxygen-induced retinopathy (Brooks et al., 2001). It has been reported that high glucose treatment stimulated endothelial cell death in isolated hearts (Ceriello et al., 2002), in cultured endothelial cells (Morishita et al., 1997; Nakagami et al., 2001; Du et al., 1999; Zou et al., 2002) and in retinas of diabetic mice and patients (Kern et al., 2000; Mohr et al., 2002). Moreover, we have shown that increased formation of peroxynitrite mediates apoptosis in endothelial cells cultured in high oxygen (Gu et al., 2003). However, molecular mechanisms underlying diabetes-induced peroxynitrite formation and acceleration of apoptosis in endothelial cells have not been elucidated. The

paradox between increases in VEGF expression and retinal cell death under diabetic condition has prompted us to study the effects of high glucose-induced oxidative stress and exogenous peroxynitrite on cell death in retinal endothelial cells in order to define the molecular mechanism by which peroxynitrite alters the survival signaling pathway of VEGF under high glucose conditions.

Materials and Methods

Cells

Retinal endothelial cells were isolated from bovine retinas by homogenization and a series of filtration steps following an established protocol in our laboratory (Behzadian et al., 1998). The cells (passages 6-8) were plated on gelatin-coated dishes or 4-well chamber slides, grown to confluence and then treated for 5 days in conditions of normal glucose (NG, 5 mM glucose) or high glucose (HG, 25 mM glucose). Various osmotic control agents including L-glucose, a glucose stereoisomer (LG, 25 mM) and 3-methyl-O-glucose, a glucose analogue that is transported into the cells but not metabolized (3mG, 25 mM) were examined. Cells were cultured in NG, HG, LG or 3mG serum-containing media for the initial 3 days of treatment and then cells were washed with PBS twice and switched to corresponding serum free media for 2 days. Serum starvation is known to induce apoptosis in endothelial cells (Gratton et al., 2001). Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY), with the exception of fetal bovine serum (FBS), which was purchased from HyClone Laboratories, Inc (Logan, UT) and CS-C complete medium, which was from Cell Systems Corporations (Kirkland, WA). Inhibitors including L-NAME, uric acid, and superoxide dismutase-polyethylene glycol (SOD-PEG) were obtained from Sigma-Aldrich (Saint Louis, MO). Inhibitors for peroxynitrite (FeTPPS) and for p38 MAP kinase (SB203580) were obtained from Calbiochem (La Jolla, CA).

Peroxynitrite treatment

Cells were switched to serum free medium, treated with 0.5 mM peroxynitrite and cultured for 16 hours. Peroxynitrite was purchased from Upstate Biotechnology (Lake Placid, NY). Stock concentrations of peroxynitrite were freshly prepared in 0.3 N NaOH. Peroxynitrite concentration was determined by spectrophotometry as described by Zou et al. (Zou et al., 2003). An equal amount of 0.3 N NaOH or decomposed peroxynitrite was used for control experiments. Neither of these had an effect on any of the parameters analyzed.

Caspase-3 activity

Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of apoptosis. The activity of the enzyme was determined using a kit from R&D systems (Minneapolis, MN) according to the manufacturer's recommendation. Briefly cells were lysed on ice for 10 minutes with lysis buffer provided with the kit. To 50 µl of cell lysate, 50 µl of 2× reaction buffer were added followed by 5 µl of caspase-3 fluorogenic substrate (DEVD-AFC). The mixture was incubated for 2 hours at 37°C and fluorescence was measured with a Cytofluor-4000 spectrophotometer (Foster City, CA) with an excitation of 400 nm and an emission of 505 nm. The assay was done with non-induced cells (serum-supplemented cells) for comparative analysis.

Hoechst stain

Apoptotic cells in cultures treated as described above were detected by nuclear staining with Hoechst 33258 (2.5 µg/ml in PBS). Hoechst 33258 was purchased from Molecular Probes (Eugene, OR). Cells were reacted with the stain for 30 minutes at room temperature, washed twice with PBS, examined and photographed in a Zeiss Axioskop microscope. At least 15 fields were counted for each treatment. Each field contained over 130 cells. Cells with condensed chromatin, shrunken, irregular or fragmented nuclei were considered apoptotic. The number of apoptotic nuclei was calculated relative to the total number of nuclei. Total cell density was also calculated for each treatment condition as an indicator of potential differences in cytotoxicity.

Western blotting analysis

Retinal endothelial cells were harvested after various treatments and lysed in modified RIPA buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 1 mM $Na_4P_2O_7$, and 1 mM PMSF) for 30 minutes on ice. Insoluble material was removed by centrifugation at 12,000 *g* at 4°C for 30 minutes. Antibodies for phospho-p38 MAP kinase, p38 MAP kinase, phospho-Akt-1, Akt-1, PARP and Akt-1 kinase kit were purchased from Cell Signaling Technology Inc (Beverly, MA). For analysis of cleaved poly ADP-ribose polymerase (PARP), phospho-p38 MAP kinase, p38 MAP kinase, phospho-Akt-1 or Akt-1, 50 µg of total protein was boiled in Laemmli sample buffer, separated on a 10-12% SDS-polyacrylamide gel by electrophoresis, transferred to nitrocellulose and reacted with specific antibody. The primary antibody was detected using a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Amersham Biosciences, Buckinghamshire, UK) and enhanced chemiluminescence. Intensity of immunoreactivity was measured using densitometry.

Immunoprecipitation

Retinal endothelial cells were cultured in high glucose in the presence or absence of peroxynitrite decomposition catalyst, or treated with peroxynitrite for the desired time. Cell lysates were prepared as described above for immunoblotting. Antibodies for nitrotyrosine, p85 and p110 subunits of PI 3-kinase were purchased from Upstate Biotechnology (Lake Placid, NY). For PI 3-kinase tyrosine nitration, 500 µg protein was incubated with p85. The precipitated proteins were analyzed by SDS-PAGE and blotted with nitrotyrosine antibody or p85 for equal loading. To study the effects on the interaction of the regulatory subunit with the catalytic subunit of PI 3-kinase, 500 µg protein was incubated with p110. The precipitated proteins were analyzed by SDS-PAGE and blotted by p85 antibody or p110 antibody for equal loading.

Akt-1 kinase assay

Retinal endothelial cells were cultured in high glucose in the presence or absence of ROS inhibitors, or treated with peroxynitrite for the desired time. For immunoprecipitation, 500 µg protein was incubated with immobilized Akt-1G1 (Cell Signaling Technology, Beverly, MA) monoclonal antibody-containing beads overnight at 4°C with gentle rocking. The beads were washed twice with lysis buffer and once with kinase buffer (25 mM Tris, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). Beads were further incubated in 40 µl kinase buffer supplemented with 200 µM ATP and 1 µg GSK-3 fusion protein for 30 minutes at 30°C. The reaction was terminated with 20 µl 3× SDS sample buffer. After boiling and centrifuging, the supernatant was separated on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane and western immunoblotting was carried out with phopho-GSK-3α/β (Ser21/9) antibody as the primary antibody. Horseradish peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence were used to detect the primary antibody. Intensity of the immunoreactivity was measured using densitometry.

Adenoviral constructs

β-Galactosidase and C-terminal HA-tagged constitutively active Akt (myr-Akt) were generated as described previously (Fulton et al., 1999). Retinal endothelial cells were infected with adenovirus [multiplicity of infection (m.o.i.) of 100] containing the β-galactosidase and myr-Akt. The virus was removed and cells were left to recover for 12 hours in complete medium. These conditions resulted in uniform expression of the transgenes in close to 95% of the cells (determined by infection with β-galactosidase, followed by staining for β-galactosidase activity). Western blot analysis using antibodies against phospho Akt-1 and GSK-3 (Cell Signaling Technology, Inc. Beverly, MA) confirmed the over-expression and activity of Akt-1 in cells transfected with myr-Akt but not in cells transfected with β-galactosidase.

Statistical analysis

The results are expressed as mean ± s.e.m. Differences among experimental groups were evaluated by ANOVA and the significance of differences between these groups was assessed by Fisher's post-hoc least significant difference test. Significance was defined at $P<0.05$.

Results

High glucose-induced peroxynitrite inactivates VEGF pro-survival signal and induces apoptosis of endothelial cells

Our previous studies in retinal endothelial cells showed that cultures maintained in high glucose had significant incr[eases] in superoxide anion, nitric oxide and peroxynitrite forma[tion] (El-Remessy et al., 2003a). In the present study, we exami[ned] the effects of high glucose-induced oxidative stress a[nd] exogenous peroxynitrite on VEGF pro-surviv[al fu]nction. As shown in Fig. 1, high glucose and per[oxy]nitrite [tre]atment accelerated serum starvation-induced [en]dothelial [cell] death as indicated by significant increas[es in] caspa[se-3] act[ivity] compared to normal glucose. T[reat]ment [with] vehicle or decomposed peroxynitrite had [no] effect on cas[pa]se-3 activity (data not shown). Caspase-[3 is] an intracellul[ar] cysteine protease that exists as a pro-enzy[me] becoming activated during the cascade of intracellular [sig]naling events that culminates in apopt[osis]. VEGF (40 ng/[ml]) inhibited serum starvation-induced activation of caspase-3 in control cultures maintained in n[orm]al gluc[ose], but had no effect on cultures treated with either high glucose or exogenous peroxynitrite. Moreover, [a] specific peroxynitrite decomposition catalyst FeTPPS (2.5 μ[M]) restored the pro-survival effects of VEGF on cultures maintained in high glucose. This finding suggests that the effect of high glucose in increasing apoptosis in retinal endothelial cells may be due to the action of peroxynitrite in altering the VEGF cell survival pathway.

Osmotic stress contributes to oxidative stress-induced cell death

Our previous studies have shown that osmotic control agents including the glucose stereoisomer (LG) and the glucose analog, 3-O-methyl glucose (3mG) also cause increases of superoxide anion and peroxynitrite formation in retinal endothelial cells, though to a lesser extent than high glucose. This effect was found to be due to activation of aldose reductase, a rate-limiting step in the polyol pathway (El-Remessy et al., 2003a). As shown in Table 1, caspase-3 activity was increased in cells cultured in high osmolarity (5 mM glucose + 20 mM LG or 5 mM glucose + 20 mM 3mG) compared to cells cultured in normal glucose. The effects of high glucose/osmotic stress were blocked by the specific aldose reductase inhibitor Zopolrestat (10 μM) and by the peroxynitrite scavengers uric acid (1 mM) and FeTPPS (2.5 μM). The caspase-3 activity was also blocked by inhibiting NOS using L-NAME (0.5 mM) and [sig]nificantly reduced by superoxide dismutase (SOD, 100 U/ml) confirming the involvement of peroxynitrite in high glucose and high osmolarity-induced cell death.

The oxidative stress-induced cell death was additionally

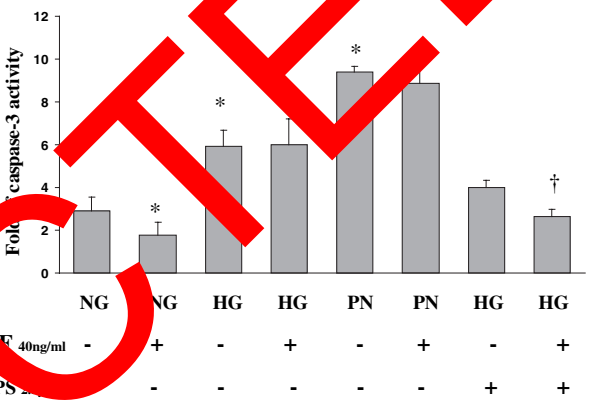

Fig. [illegible]h glucose and peroxynitrite inactivate VEGF pro-survival and accelerate endothelial cell death. Statistical analysis of caspase-3 activity showing significant increases in apoptosis in cells treated with peroxynitrite (PN) or cultured in high glucose (HG) compared to normal glucose (NG). Exogenous VEGF (40 ng/ml) protected cells cultured in normal glucose from serum starvation-induced apoptosis but did not rescue cells cultured in high glucose or peroxynitrite. FeTPPS (2.5 μM) a specific peroxynitrite decomposition catalyst restored the pro-survival effect of VEGF in cells maintained in high glucose cultures. Similar results were obtained in another three experiments. (*$P<0.05$ compared to NG, $P<0.05$ compared to HG.)

Table 1. Retinal endothelial cell caspase-3 activity as a measure of apoptosis induced in high glucose and high osmolarity is blocked by inhibiting the polyol pathway and by blocking peroxynitrite formation

	Caspase-3 activity (fold)			
Treatment	NG	HG	3mG	LG
Untreated	4.6±0.45	8.1±0.7*	7.15±0.6*	7.6±0.5*
Zoplorestat	3.6±0.26	4.9±0.29	4.7±0.27	4.6±0.34
Uric acid	3.6±1.34	4.6±0.5	5.1±0.4	4.8±0.7
FeTTPS	3.4±0.31	3.85±0.4	3.7±0.21	ND
L-NAME	3.93±0.3	3.6±0.3	3.5±0.2	ND
SOD	3.8±0.37	5.6±0.6†	5.9±0.5†	5.8±0.6†

NG, normal glucose (5 mM); HG, high glucose (25 mM); 3mG, 3-O-methyl glucose (5 mM glucose+20 mM 3mG); LG, L-glucose (5 mM glucose+20 mM L-glucose)

Zopolrestat (10 μM), an aldose reductase inhibitor; FeTPPS (2.5 μM) and uric acid (1 mM), peroxynitrite scavengers; L-NAME (0.5 mM), a NOS inhibitor; SOD (100 U/ml) superoxide dismutase.

*$P<0.05$ compared with NG; †$P<0.05$ compared with untreated HG, 3mG or LG.

Table 2. High glucose- and high osmolarity-induced apoptosis of retinal endothelial cells is blocked by inhibiting peroxynitrite formation

	% Apoptotic cells			
Treatment	NG	HG	3mG	LG
Untreated	16.8±1.5	31.4±1.9*	28.1±2.5*	25.8±2.1*
Uric acid	15.7±1.3	19.5±1.7	20.2±1.7	19.7±1.7
L-NAME	14.6±1.3	20.1±1.9	19.65±1.8	17.6±1.5
SOD	15.7±1.4	26.3±2.3†	22.9±2.1†	21.5±1.7†

Percentage of apoptotic cells were calculated by counting 10 fields, 200 cells each in at least three independent cultures.

NG, normal glucose (5 mM); HG, high glucose (25 mM); 3mG, 3-O-methyl glucose (5 mM glucose+20 mM 3mG); LG, L-glucose (5 mM glucose+20 mM L-glucose)

Uric acid (1 mM) a peroxynitrite scavenger; L-NAME (0.5 mM), a NOS inhibitor; SOD (100 U/ml), superoxide dismutase.

*P<0.05 compared with NG; †P<0.05 compared with untreated HG, 3mG or LG.

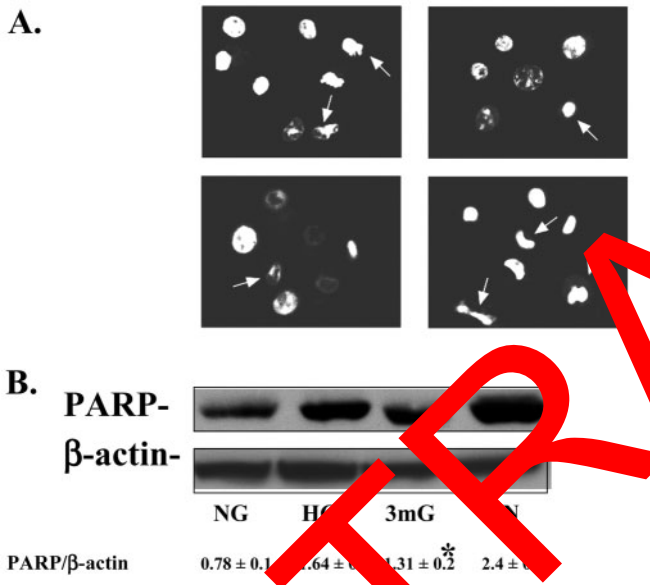

Fig. 2. Oxidative stress-induced apoptosis in high glucose, high osmolarity and peroxynitrite. (A) Representative images of Hoechst 33258 nuclear staining show apoptotic cells that were identified based on their shrunken, irregular or fragmented nuclei. Quantification of these data is shown in Table 2. (B) A representative immunoblot, using cell lysates (50 μg), for cleaved PARP and β-actin. Below: statistical analysis of quantification of cleaved PARP showing significant increases in apoptosis in cells cultured with high glucose (HG), 3-O-methyl glucose (3mG) or peroxynitrite in comparison to cells cultured in normal glucose (*P<0.05 vs NG.)

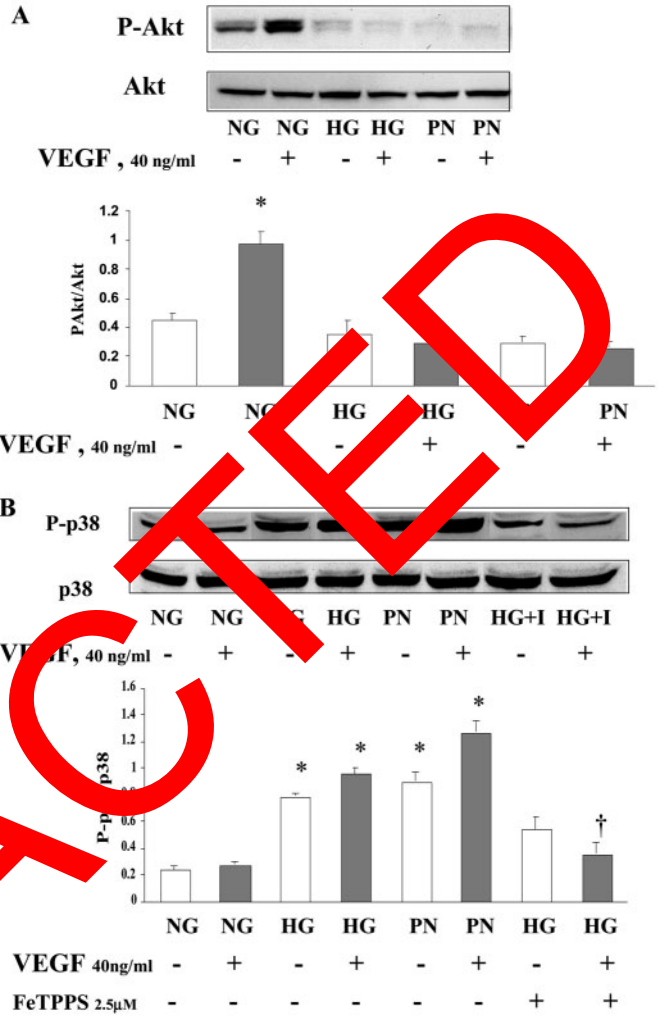

Fig. 3. High glucose and peroxynitrite inactivate VEGF pro-survival via alteration of Akt-1/P38 MAP kinase activation. (A) High glucose (HG) and peroxynitrite (PN) significantly decreased phosphorylation of Akt-1 compared to normal glucose (NG) even in the presence of exogenous VEGF (40 mg/ml). (*P<0.05 compared to NG.) (B) High glucose (HG) and peroxynitrite (PN) significantly increased phosphorylation of p38 MAP kinase in basal or VEGF (40 mg/ml)-stimulated conditions. FeTPPS (2.5 μM) a specific peroxynitrite decomposition catalyst blocked the increases in p38 MAP kinase phosphorylation in both basal and VEGF stimulated conditions. Identical results were obtained in another two experiments. (*P<0.05 compared to NG, †P<0.05 compared to HG.)

assessed by nuclear staining with Hoechst 33258 which showed that cultures maintained in medium with high glucose or high osmolarity (3mG or LG) had significantly increased numbers of cells with shrunken or irregular nuclei characteristic of apoptosis (32%, 28% and 26% respectively, P<0.01, Table 2). Fig. 2A shows images of Hoechst nuclear stain of cells undergoing apoptosis. The increase in apoptotic cell profiles was significantly inhibited by treatment with uric acid, superoxide dismutase, or L-NAME providing further support for the role of peroxynitrite in the apoptotic process. The oxidative stress-induced cell apoptosis was further confirmed using western blot analysis of cleaved poly ADP-ribose polymerase (PARP). Detection of cleaved PARP (89 kDa), a cleavage target of caspase-3, is accepted as a marker for cell apoptosis. A representative western blot and normalized PARP/actin expression are shown in Fig. 2B. Cultures maintained in high glucose, 3-O-methyl glucose or exogenous peroxynitrite showed significant increases in PARP expression compared to cultures maintained in normal glucose.

High glucose-induced peroxynitrite inactivates VEGF pro-survival via alteration of Akt-1/P38 MAP kinase

The above data indicate that peroxynitrite-mediated oxidative

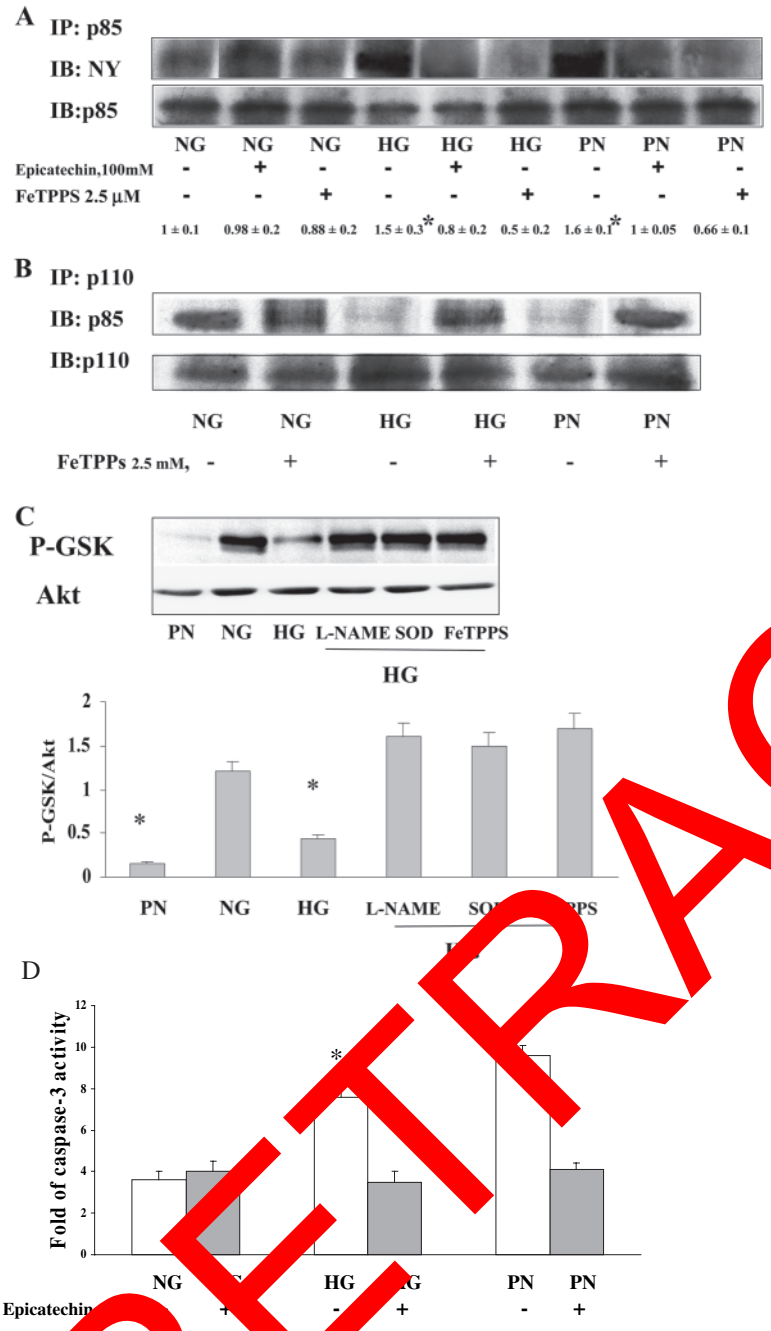

Fig. 4. Tyrosine nitration inactivates PI 3-kinase/Akt-1 pro-survival pathway. (A) Immunoprecipitation with p85 subunit of PI 3-kinase and western blot analysis using anti-nitrotyrosine antibody showed that cells treated with high glucose (HG) or peroxynitrite (PN) had significantly more nitration on the regulatory p85 subunit compared with cells cultured in normal glucose (NG). (*P<0.05 compared to NG.) This effect was blocked by the specific peroxynitrite decomposition catalyst FeTPPS (2.5 μM) and the specific nitration inhibitor epicatechin (100 μM). (B) Immunoprecipitation with p110 subunit of PI 3-kinase and western blot analysis using p85 antibody showed that under high glucose (HG) or peroxynitrite (PN) treatments, the p85 subunit was hardly detected in the immunopreicipitate of the catalytic p110 subunit compared with cells cultured in normal glucose (NG). Cells were stimulated with VEGF (40 ng/ml) in the presence or absence of the peroxynitrite decomposition catalyst (FeTPPs). The association between p85 and p110 was restored by treatment with FeTPPs (2.5 μM). (C) High glucose (HG) and peroxynitrite (PN) decreased Akt-1 activity significantly compared to normal glucose (NG). Akt-1 kinase activity was restored by treatment of high glucose cultures with the specific peroxynitrite inhibitor (2.5 μM), NOS inhibitor (L-NAME, 0.5 mM) and superoxide dismutase (SOD, 100 U/ml). A western blot of phospho GSK-3, the substrate of Akt-1 kinase is shown at the top. (*P<0.05 compared to NG.) (D) Statistical analysis of caspase-3 activity showing significant increases in apoptosis of cells cultured in high glucose (HG) or treated with peroxynitrite (PN) compared to normal glucose (NG). These effects were blocked by the specific nitration inhibitor epicatechin (100 μM). Similar results were obtained in another three experiments. (*P<0.05 compared to NG.)

stress accelerates retinal endothelial cell death in cultures maintained in high glucose and inactivates VEGF pro-survival function. Activation of the PI 3-kinase/Akt-1 pathway plays a critical role in the VEGF-survival pathway, whereas activation of p38 MAP kinase can induce cell death. We, therefore, examined whether the high glucose-induced impairment of VEGF-mediated cell survival function could be the result of alterations of the pro-survival Akt-1 pathway or the pro-apoptotic p38 MAP kinase pathway. Analysis of the VEGF-induced phosphorylation of Akt-1 showed that the pro-apoptotic effect of either high glucose-induced oxidative stress or exogenous peroxynitrite was associated with significant decreases in phosphorylation of Akt-1 in basal and VEGF-stimulated conditions (Fig. 3A). Analysis of the VEGF-induced phosphorylation of P38 MAP kinase showed that the pro-apoptotic effect of either high glucose or peroxynitrite was associated with significant increases in phosphorylation of p38 MAP kinase in the presence or absence of exogenous VEGF (Fig. 3B). Treatment of cultures maintained in high glucose with the specific peroxynitrite decomposition catalyst FeTTPS blocked the increases in p38 MAP kinase phosphorylation in both basal and VEGF-stimulated conditions. Control experiments with decomposed peroxynitrite showed no significant difference compared to normal glucose (data not shown).

Tyrosine nitration inactivates PI 3-kinase/Akt-1 pro-survival pathway

Previous work in vitro has shown that the p85 regulatory subunit of PI 3-kinase is one target for peroxynitrite-induced protein nitration on tyrosine (Hellberg et al., 1998). Our immunoprecipitation studies with an antibody that recognizes the p85 regulatory subunit of PI 3-kinase showed that cultures maintained in high glucose or treated with peroxynitrite had a significant increase in tyrosine nitration of p85 compared to cultures in normal glucose (Fig. 4A). These increases in tyrosine nitration were blocked by the specific peroxynitrite decomposition catalyst FeTTPs (2.5 μM) and by the specific nitration inhibitor epicatechin (100 μM). Epicatechin, a dietary flavanol, has been used to selectively block the nitrating effect of peroxynitrite on tyrosine residues (Schroeder et al., 2001;

Schroeder et al., 2003). In order to investigate the effect of tyrosine nitration in causing PI 3-kinase dysfunction, we determined the effects of high glucose and exogenous peroxynitrite on the association between the regulatory subunit of p85 and the catalytic subunit p110 in the presence or absence of peroxynitrite decomposition catalyst (FeTPPs, 2.5 μM). We stimulated cells with VEGF (40 ng/ml) and immunoprecipitated the p110 catalytic subunit and detected the presence of p85 in normal glucose controls (Fig. 4B). However, in treatments with high glucose or peroxynitrite, p85 could no longer be detected in the immunoprecipitates. These results indicate the dissociation of p85 from p110, because reprobing the blots with p110 antibodies revealed the presence of p110. Treatments with FeTPPs restored the association between p85 and p110 in cultures of high glucose or peroxynitrite-treated cells and did not affect normal glucose controls. These results suggest that $ONOO^-$ could modulate the cell survival responses mediated by PI 3-kinase activation.

In order to further investigate the physiological role of nitration of the PI 3-kinase subunits, we determined the effects of high glucose and exogenous peroxynitrite on the activity of Akt-1 kinase, a downstream target of PI 3-kinase in the survival pathway. Analysis of Akt-1 kinase activity in phosphorylating GSK-3 showed that both high glucose and exogenous peroxynitrite inhibited Akt-1 kinase activity. The effect of high glucose was reversed by the NOS inhibitor (L-NAME, 0.5 mM), superoxide dismutase (SOD, 100 U/ml) or specific peroxynitrite decomposition catalyst FeTPPS (2.5 μM, Fig. 4C).

To evaluate the causal relationship between the nitration of PI 3-kinase and the pro-apoptotic effects of high glucose and peroxynitrite, we tested the effects of the nitration inhibitor epicatechin (100 μM) in blocking activation of caspase-3. These experiments showed that inhibiting tyrosine nitration completely blocks the pro-apoptotic effects of high glucose or exogenous peroxynitrite as indicated by reduced caspase-3 activity (Fig. 4D). We verified the effects of blocking PI 3-kinase on blocking cell survival and inducing cell apoptosis by measuring caspase-3 activity. Cells were treated with the specific PI 3-kinase inhibitors wortmannin (100 nM) or LY294002 (10 μM) in the presence or absence of VEGF (40 ng/ml) and the activity of caspase-3 was determined. Under basal condition, treatments with wortmannin or LY294002 resulted in significant increases in caspase-3 activity (12.7±1.1 and 14.1±1.1 fold compared to 3.6±0.8 fold in control). Stimulation with VEGF rescued control cells and significantly reduced caspase-2 activity (1.7±0.2). However, in cells treated with wortmannin or LY294002, VEGF did not rescue cells and the activity of caspase-3 was significantly increased (13.1±1.1 and 16.9±1.7). Together, these findings confirm the correlation between the inhibitory effects of PI 3-kinase nitration, the decreases in Akt-1 activity and the pro-apoptotic effects of high glucose and peroxynitrite.

Transfection of active Akt-1 restores cell survival and inhibits phosphorylation of p38MAP kinase

The above data imply that the p38 MAP kinase-driven pro-apoptotic pathway becomes important when the PI 3-kinase/Akt-1 pro-survival pathway is inhibited. To confirm the role of PI 3-kinase/Akt-1 inhibition in pro-apoptotic effects of high glucose or peroxynitrite, we used adenoviral-mediated gene transfer of constitutively active Akt-1 (myr-Akt) into retinal endothelial cells. Fig. 5A shows that infection with a myr-Akt blocked the high glucose- or peroxynitrite-induced apoptosis as compared to control cells transfected with β-galactosidase constructs. Moreover, the anti-apoptotic effect of myr-Akt was associated with significant decreases in phosphorylation of p38 MAP kinase in high glucose or peroxynitrite-treated cultures compared to cultures infected with β-galactosidase constructs. Fig. 5B shows a representative image of phospho p38 MAP kinase western blot and statistical analysis of three independent experiments.

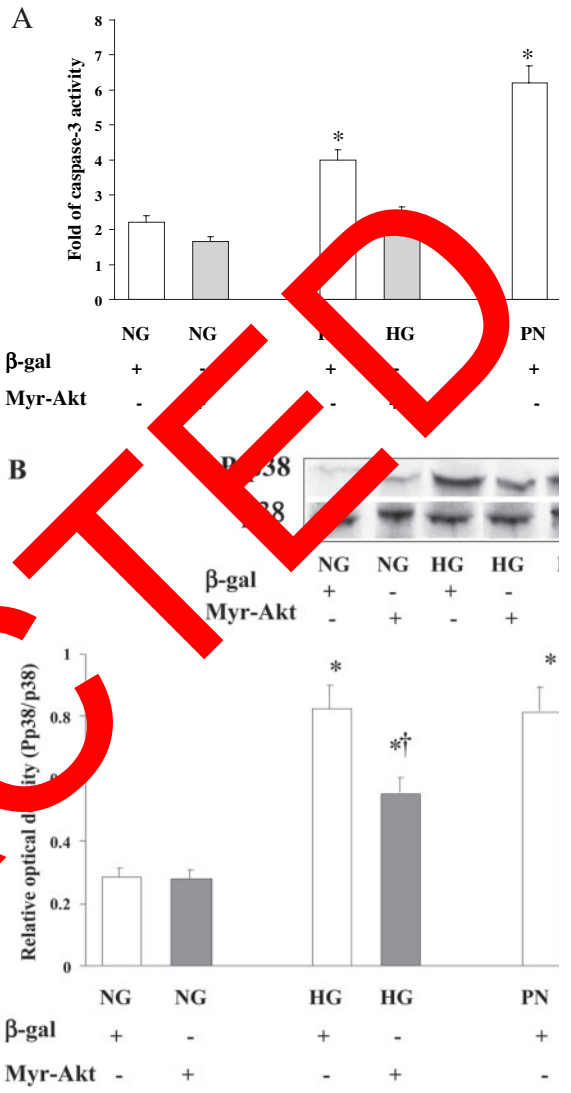

Fig. 5. Transfection of active Akt-1 restores cell survival and inhibits p38 MAP kinase phosphorylation. (A) Statistical analysis of caspase-3 activity shows that transfection with myr-Akt-1 significantly blocks increases in caspase-3 activity in cells cultured in high glucose (HG) or peroxynitrite (PN) compared to cells transfected with β-galactosidase (β-gal). (*P<0.05 compared to β-gal.) (B) Transfection with myr-Akt-1 decreases p38 MAP kinase phosphorylation in cells cultured in high glucose (HG) or peroxynitrite (PN) compared to cells transfected with β-galactosidase. A representative image of immunoblot analysis of phospho p38 MAP kinase and β-actin is shown at the top. Similar results were obtained in another two experiments. (*P<0.05 compared to NG, $^{\dagger}P$<0.05 compared to HG and PN.)

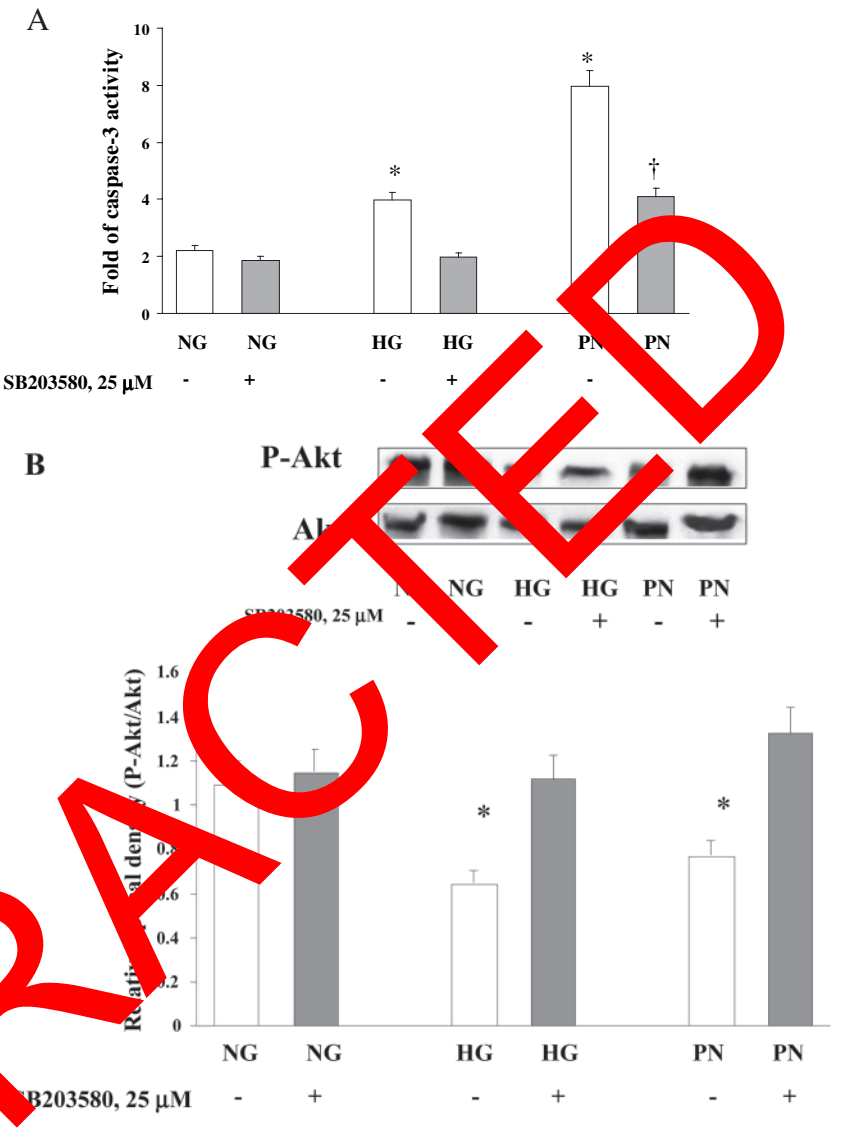

Fig. 6. Inhibition of p38 MAP kinase with SB203580 restores cell survival and Akt-1 phosphorylation. (A) Statistical analysis of caspase-3 activity shows that inhibition of p38 MAP kinase significantly reduced increases of caspase-3 activity in cells cultured in high glucose (HG) or peroxynitrite (PN) compared to cells cultured in normal glucose (NG). (*P<0.05 compared to NG, †P<0.05 compared to PN.) (B) Inhibition of p38 MAP kinase restores Akt-1 phosphorylation in cells cultured in high glucose (HG) or peroxynitrite (PN) compared to cells cultured in normal glucose (NG). A representative image of immunoblot analysis, using cell lysates (50 μg), for phospho Akt-1 and β-actin is shown at the top. Similar results were obtained in another two experiments. (*P<0.05 compared to NG.)

Inhibition of p38 MAP kinase restores cell survival and Akt-1 phosphorylation

To confirm the role of p38 MAP kinase activation in the pro-apoptotic effects of high glucose or exogenous peroxynitrite, we treated the cells with the specific p38 MAP kinase inhibitor SB203580 (25 μM) and assessed apoptosis by measurement of caspase-3 activity. The p38 MAP kinase inhibitor did not affect cell death in the normal glucose control but reduced significantly the apoptosis induced in high glucose-maintained or exogenous peroxynitrite-treated cultures (Fig. 6A). Moreover, inhibition of p38 MAP kinase restored basal levels of Akt-1 phosphorylation in high glucose [illegible] peroxynitrite-treated cultures, but ha[illegible] no significant effect on normal cultures (F[illegible] 6B). This result suggests that cross-talk be[illegible]I 3-kinase/Akt-1 and p38 MAP ki[illegible]e signal pathways contributes to the ba[illegible]ce of pro- v[illegible] anti-apoptotic signaling path[illegible]y [illegible]d hence the survival of endothelial cells.

Discussion

The present stu[illegible] provide[illegible] a novel mechanism of oxidative stress-induced ap[illegible]tos[illegible] via ty[illegible]ine nitration-mediated inhibition [illegible]PI-3kin[illegible]/Akt-1 [illegible] activation of p38 MAP kinase [illegible]athway. In this [illegible]udy [illegible]e demonstrate that tyrosine nitra[illegible]n of the [illegible] 3-kinase [illegible] subunit inactivates the survival sig[illegible]ling path[illegible] retinal endothelial cells cultured in high gluco[illegible] or [illegible]xogenous peroxynitrite. These effects are blocked by the [illegible]cific peroxynitrite decomposition catalyst FeTPPs and by the [illegible]ecific nitration inhibitor epicatechin. We show also for the [illegible]t time that the pro-apoptotic effect of high glucose or peroxynitrite is associated with imbalance between Akt-1 and p38 MAP kinase phosphorylation and that blocking p38 MAP kinase or over-expression of constitutively active Akt-1 masks the pro-apoptotic effect of high glucose or peroxynitrite and restores survival function in retinal endothelial cells.

High glucose concentrations mimicking the diabetic situation have been shown to trigger endothelial cell death (Baumgartner-Parzer et al., 1995; Du et al., 1999; Ido et al., 2002; Mizutani et al., 1996; Mohr et al., 2002; Quagliaro et al., 2003) and to increase formation of reactive oxygen and nitrogen species (Cosentino et al., 1997; El-Remessy et al., 2003a; Quagliaro et al., 2003; Zou et al., 2002). Paradoxically, high glucose also stimulates formation of the endothelial cell survival factor VEGF (Behzadian et al., 2003; Kim et al., 2000; Williams et al., 1997). Thus, we tested the hypothesis that high glucose increases apoptosis in endothelial cells through the action of peroxynitrite altering the VEGF cell survival pathway. We examined the pro-survival effects of exogenous VEGF (40 ng/ml) on rescuing endothelial cells from serum withdrawal-induced apoptosis under various conditions by determining caspase-3 activity. VEGF protected endothelial cells cultured in normal glucose but not those cultured in high glucose or peroxynitrite. The peroxynitrite decomposition catalyst (FeTPPs) restored the pro-survival effect of VEGF in cells cultured in high glucose, suggesting that high glucose alters pro-survival signaling of VEGF via peroxynitrite formation. Cell death by apoptosis was also confirmed by morphological study of nuclear changes, using the nuclear stain Hoechst 33258 and western blot analysis of cleaved poly

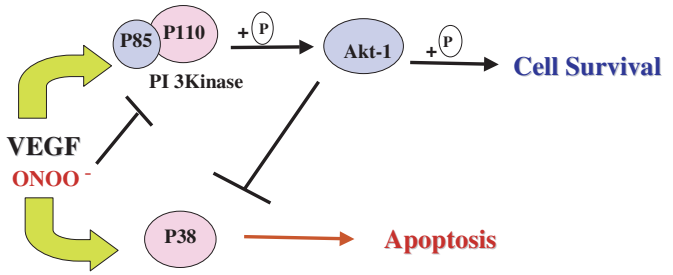

Fig. 7. A schematic representation of the proposed mechanism by which high glucose, via its effect on peroxynitrite, inactivates the VEGF/PI 3-kinase/Akt-1 pro-survival pathway and stimulates cell death via activation of p38 MAP kinase pathway. Nitration of PI 3-kinase is proposed as a mechanism by which high glucose switches off the VEGF pro-survival pathway and triggers the pro-apoptotic pathway.

(ADP-ribose) polymerase (PARP). The pro-apoptotic effects of high glucose and peroxynitrite were associated with activation of p38 MAP kinase in the presence or absence of exogenous VEGF. These results are in agreement with previous reports (Igarashi et al., 1999; Nakagami et al., 2001). Moreover, the peroxynitrite decomposition catalyst FeTPPS blocked the increases in p38 MAP kinase phosphorylation in high glucose cultures under basal or VEGF-stimulated conditions, further supporting a causal role of peroxynitrite inactivating VEGF pro-survival signal and causing cell death.

The role of the PI 3-kinase/Akt-1 pathway in VEGF's pro-survival function was confirmed by studies showing that specific inhibitors of PI 3-kinase, including wortmanin and LY294002, caused retinal endothelial cells to undergo apoptosis as indicated by significant increases in caspase-3 activity in the presence or absence of exogenous VEGF. These results are in agreement with several reports showing VEGF does not rescue endothelial cells from serum starvation-induced apoptosis in the presence of a specific inhibitor of PI 3-kinase (Fujio and Walsh, 1999; Gerber et al., 1998; Gratton et al., 2001; Suhara et al., 2001). Previous work in vitro has shown that the p85 regulatory subunit of PI 3-kinase is a target for peroxynitrite-induced tyrosine nitration (Hellberg et al., 1998). Our data showed that cultures treated with high glucose or exogenous peroxynitrite showed significant increases in tyrosine nitration of the p85 subunit. This effect was accompanied by dramatic decreases in the association of the regulatory p85 subunit from the catalytic p110 subunit, as well as by significant decreases in Akt-1 phosphorylation and Akt-1 kinase activity, confirming an inhibitory effect of the nitration of PI 3-kinase. Moreover, the specific peroxynitrite decomposition catalyst FeTPPs and the nitration inhibitor epicatechin blocked tyrosine nitration, restored p85/p110 interaction of PI 3-kinase and Akt-1 kinase and survival promoting activity. These results confirm the relationship between nitration of p85, the decreases in Akt-1 activity and the pro-apoptotic effects of high glucose and peroxynitrite. Akt-1 kinase activity was restored also by inhibiting NOS and by SOD. Our findings lend further support to previous reports of significant increases in tyrosine nitration in diabetic patients (Ceriello et al., 2001), in high glucose models (Ceriello et al., 2002; Zou et al., 2002) and in experimental diabetes (El-Remessy et al., 2003b; Turko et al., 2001). However, our study is the first we know of to elucidate the molecular mechanism of inhibitory tyrosine nitration of p85 leading to inactivation of the pro-survival effects of VEGF/Akt-1in a physiological model of high glucose-induced oxidative stress.

Further work is needed to determine the effects of high glucose and peroxynitrite-induced tyrosine nitration on other steps in the VEGF signal transduction pathway. Studies now in progress in our lab indicate that high glucose and peroxynitrite also induce tyrosine nitrosylation of VEGFR2. Interestingly, the high glucose treatment also causes increases in tyrosine phosphorylation of VEGFR2. This suggests that tyrosine nitration induces activation of VEGFR2, as has been shown previously for PDGF and EGF (Kim et al., 2000; van der Vliet et al., 1998). More work is needed to define the specific mechanism of VEGFR2 activation by high glucose and to determine the molecular and physiological consequences. However, this observation suggests that the inhibitory effects of high glucose and peroxynitrite on cell survival are specific to a direct action of the tyrosine nitration inhibiting the PI 3K/Akt signaling pathway.

Parallel activation of survival and death pathways has recently been documented in response to VEGF, FGF and cytokines such as TNF-α (Cardier and Erickson-Miller, 2002; Gratton et al., 2001; Harfouche et al., 2003; Matsumoto et al., 2002). Thus, it is likely that high glucose and peroxynitrite-induced alterations in the Akt-1 and p38 MAP kinase interaction could alter cell responses to other cell survival stimuli in addition to VEGF. While further study is needed to directly test this hypothesis, our previous study has shown that peroxynitrite blocks the effects of either basic FGF or serum in activating the PI 3-kinase/Akt cell survival pathway (Gu et al., 2003).

We investigated the interaction between Akt-1 and p38 MAP kinase pathways under high glucose or exogenous peroxynitrite treatment. It is interesting that expression of constitutively active Akt-1 (myr-Akt-1) masked the pro-apoptotic effects of high glucose and exogenous peroxynitrite and restored cell survival function in treated cells. In addition, blocking of p38 MAP kinase also rescued retinal endothelial cells from accelerated cell death. The protective effects of SB203580 were more prominent in high glucose cultures than in peroxynitrite-treated cells, probably because of the magnitude of peroxynitrite-induced cell death. Our results are in agreement with previous reports in other vascular endothelial cells (Fujio and Walsh, 1999; Gratton et al., 2001; Harfouche et al., 2003; Matsumoto et al., 2002; Yue et al., 1999). Furthermore, the anti-apoptotic effect of myr-Akt-1 was associated with significant inhibition of p38 MAP kinase phosphorylation and the protective effect of SB203580 was associated with significant increases in Akt-1 phosphorylation. (see Figs 5 and 6). These findings demonstrate for the first time a cross talk between Akt-1 and the p38 MAP kinase signaling pathway in retinal endothelial cells. It has been reported that inhibition of p38 activation is mediated through phosphorylation and inhibition of MEKK3 by Akt-1 in aortic endothelial cells (Gratton et al., 2001). Similar results were reported (Harfouche et al., 2003) in HUVEC in response to angiopoietin-1 suggesting that the negative influence of Akt-1 on p38 MAP kinase is a general phenomenon in endothelial cells.

Of note, we and others have reported the effects of osmotic

stress in increasing oxidative stress (Du et al., 1999; El-Remessy et al., 2003a; Obrosova et al., 2003; Shaw et al., 2003). Mannitol is commonly used as an osmotic control for high glucose treatment. However, it has been shown that mannitol acts as a free radical scavenger, thereby reducing intracellular levels of reactive oxygen species (El-Remessy et al., 2003a; Karasu, 2000). For osmotic control studies, we used the glucose stereoisomer (LG) and the glucose analog, 3-O-methyl glucose (3mG). Both LG and 3mG accelerated cell death though to a lesser extent than that seen with high glucose. These effects were blocked by the NOS inhibitor L-NAME, superoxide dismutase (SOD) and peroxynitrite scavengers (uric acid and FeTPPS) confirming the role of oxidative stress- and peroxynitrite-induced cell death. Moreover, blocking aldose reductase, a rate-limiting step in the polyol pathway, prevented the pro-apoptotic effects of osmotic stress in high glucose or osmotic control cultures. Similar results have been reported in vivo and in vitro (Obrosova et al., 2003; Miwa et al., 2003).

Taken together, our data suggest that the increased formation of peroxynitrite stimulated by high glucose inactivates VEGF pro-survival function in retinal endothelial cell and triggers apoptosis. In endothelial cells, VEGF promotes the parallel alteration of Akt-1 and p38 MAP kinase, anti- and pro-apoptotic pathways, respectively. It is suggested that inhibition of PI 3-kinase by peroxynitrite-induced tyrosine nitration triggers a molecular switch, reducing Akt-1 activation and facilitating the pro-apoptotic p38 MAP kinase pathway, thus initiating a molecular cascade in which VEGF loses its ability to sustain cell survival in the presence of high glucose or oxidative stress. A schematic representation of the proposed mechanism is shown in Fig. 7. The fact that the peroxynitrite decomposition catalyst FETPPS and the nitration inhibitor epicatechin restored pro-survival function in retinal endothelial cells opens the door for a possible new target in controlling early diabetic retinopathy.

This work was supported by NIH grants: NIH-EY [illegible]18; NIH-EY11766, American Heart Association (AHA) Beginning Grant in Aid and by Postdoctoral Fellowships from Association for Research in Vision and Ophthalmology/Ciba Vision Inc. and AHA. Peter Oates, Pfizer Pharmaceuticals Corp. provided Zopolrestat.

References

Aiello, L. [illegible] R. L., [illegible]igg, P. [illegible]Keyt, B. A., Jampel, H. D., Shah, S. T. [illegible]asquale, [illegible] R., Thie[illegible] Iwamoto, M. A., Park, J. E. et al. (19[illegible]). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N. Engl. J. Med.* **331**, 1480-148[illegible].

Baumgartner-Parzer, S. M., Wagner, L., Pettermann, M., Grillari, J., Gessl, A. and Waldhausl, W. (1995). High-glucose-triggered apoptosis in cultured endothelial cells. *Diabetes* **44**, 1323-1327.

Behzadian, M. A., Wang, X. L., Shabrawey, M. and Caldwell, R. B. (1998). Effects of hypoxia on glial cell expression of angiogenesis-regulating factors VEGF and TGF-beta. *Glia* **24**, 216-225.

Behzadian, M. A., El-Remessy, A., Franklin, T. and Caldwell, R. B. (2003). High glucose induces urokinase receptor (uPAR) expression in vascular endothelial cells through the GSK3/β-catenine pathway. *Invest. Ophthalmol. Vis. Sci.* **44**, 3905.

Brooks, S. E., Gu, X., Samuel, S., Marcus, D. M., Bartoli, M., Huang, P. L. and Caldwell, R. B. (2001). Reduced severity of oxygen-induced retinopathy in eNOS-deficient mice. *Invest. Ophthalmol. Vis. Sci.* **42**, 222-228.

Cardier, J. E. and Erickson-Miller, C. L. (2002). Fas (CD95)- and tumor necrosis factor-mediated apoptosis in liver endothelial cells: role of caspase-3 and the p38 MAPK. *Microvasc. Res.* **63**, 10-18.

Ceriello, A., Mercuri, F., Quagliaro, L., Assaloni, R., Motz, E., Tonutti, L. and Taboga, C. (2001). Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. *Diabetologia* **44**, 834-838.

Ceriello, A., Quagliaro, L., D'Amico, M., di Filippo, C., Marfella, R., Nappo, F., Berrino, L., Rossi, F. and Giugliano, D. (2002). Acute hyperglycemia induces nitrotyrosine formation and apoptosis in perfused heart from rat. *Diabetes* **51**, 1076-1082.

Cosentino, F., Hishikawa, K., Katusic, Z. S. and Lu[illegible] (1997). High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *[illegible]culation* **96**, 25[illegible].

Du, X., Stocklauser-Farber, K. and Rosen, P. (1999). Generation of reactive oxygen intermediates, activation of NF-kappaB, and induction of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase? *Free Radic. Biol. Med.* **27**, 752-763.

Duh, E. and Aiello, L. P. (199[illegible] Vascular endothelial growth factor and diabetes: the agonist versus antagonist paradox. *Diabetes* [illegible]1899-1906.

El-Remessy, A. B., Abou-Mohamed, G., Caldwell, R. W. and Caldwell, R. B. (2003a). High glucose-induced tyrosine nitration in endothelial cells: role of eNOS uncoupling and aldose reductase activation. *Invest. Ophthalmol. Vis. Sci.* **44**, 3135[illegible]3.

El-Remessy, A. [illegible] Behzadian, M. A., Abou-Mohamed, G., Franklin, T., Caldwell, R. W. and Caldwell, R. B. (2003b). Experimental diabetes causes breakdown of the blood-retina barrier by a mechanism involving tyrosine nitration and increased expression of vascular endothelial growth factor and urokinase plasminogen activator receptor. *Am. J. Pathol.* **162**, [illegible]5-2004.

Fu[illegible], Y. and Walsh, K. (1999). Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J. Biol. Chem.* **274**, 16349-16354.

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597-601.

[illegible] W., Antonetti, D. A., Barber, A. J., LaNoue, K. F. and Levison, S. W. (2002). Diabetic retinopathy: more than meets the eye. *Surv. Ophthalmol.* **47**, S253-S262.

Gerber, H. P., Dixit, V. and Ferrara, N. (1998a). Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J. Biol. Chem.* **273**, 13313-13316.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998b). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* **273**, 30336-30343.

Graier, W. F., Posch, K., Fleischhacker, E., Wascher, T. C. and Kostner, G. M. (1999). Increased superoxide anion formation in endothelial cells during hyperglycemia: an adaptive response or initial step of vascular dysfunction? *Diabetes Res. Clin. Pract.* **45**, 153-160.

Gratton, J. P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K. and Sessa, W. C. (2001). Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *J. Biol. Chem.* **276**, 30359-30365.

Gu, X., El-Remessy, A., Brooks, S. E., Al-Shabrawey, M., Tsai, N. T. and Caldwell, R. B. (2003). Hyperoxia induces retinal vascular endothelial cell apoptosis through formation of peroxynitrite. *Am. J. Physiol. Cell Physiol.* **285**, C546-C554.

Harfouche, R., Gratton, J.-P., Yancopoulos, G. D., Noseda, M., Karsan, A. and Hussain, S. N. A. (2003). Angiopoietin-1 activates both anti- and proapoptotic mitogen-activated protein kinases. *FASEB J.* **17**, 1523-1525.

Hellberg, C. B., Boggs, S. E. and Lapetina, E. G. (1998). Phosphatidylinositol 3-kinase is a target for protein tyrosine nitration. *Biochem. Biophys. Res. Commun.* **252**, 313-317.

Ido, Y., Carling, D. and Ruderman, N. (2002). Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes* **51**, 159-167.

Igarashi, M., Wakasaki, H., Takahara, N., Ishii, H., Jiang, Z. Y., Yamauchi, T., Kuboki, K., Meier, M., Rhodes, C. J. and King, G. L. (1999). Glucose or diabetes activates p38 mitogen-activated protein kinase via different pathways. *J. Clin. Invest.* **103**, 185-195.

Joussen, A. M., Poulaki, V., Qin, W., Kirchhof, B., Mitsiades, N., Wiegand, S. J., Rudge, J., Yancopoulos, G. D. and Adamis, A. P. (2002). Retinal vascular endothelial growth factor induces intercellular adhesion molecule-

1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. *Am. J. Pathol.* **160**, 501-509.

Karasu, C. (2000). Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: role of reactive oxygen species. *Eur. J. Pharmacol.* **392**, 163-173.

Kern, T. S., Tang, J., Mizutani, M., Kowluru, R. A., Nagaraj, R. H., Romeo, G., Podesta, F. and Lorenzi, M. (2000). Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. *Invest. Ophthalmol. Vis. Sci.* **41**, 3972-3978.

Kim, N. H., Jung, H. H., Cha, D. R. and Choi, D. S. (2000). Expression of vascular endothelial growth factor in response to high glucose in rat mesangial cells. *J. Endocrinol.* **165**, 617-624.

Klotz, L. O., Schieke, S. M., Sies, H. and Holbrook, N. J. (2000). Peroxynitrite activates the phosphoinositide 3-kinase/Akt pathway in human skin primary fibroblasts. *Biochem. J.* **352**, 219-225.

Matsumoto, T., Turesson, I., Book, M., Gerwins, P. and Claesson-Welsh, L. (2002). p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J. Cell Biol.* **156**, 149-160.

Misko, T. P., Highkin, M. K., Veenhuizen, A. W., Manning, P. T., Stern, M. K., Currie, M. G. and Salvemini, D. (1998). Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. *J. Biol. Chem.* **273**, 15646-15653.

Miwa, K., Nakamura, J., Hamada, Y., Naruse, K., Nakashima, E., Kato, K., Kasuya, Y., Yasuda, Y., Kamiya, H. and Hotta, N. (2003). The role of polyol pathway in glucose-induced apoptosis of cultured retinal pericytes. *Diabetes Res. Clin. Pract.* **60**, 1-9.

Mizutani, M., Kern, T. S. and Lorenzi, M. (1996). Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J. Clin. Invest.* **97**, 2883-2890.

Mohr, S., Xi, X., Tang, J. and Kern, T. S. (2002). Caspase activation in retinas of diabetic and galactosemic mice and diabetic patients. *Diabetes* **51**, 1172-1179.

Morishita, R., Higaki, J., Hayashi, S. I., Yo, Y., Aoki, M., Nakamur[illegible] S., Moriguchi, A., Matsushita, H., Matsumoto, K., Nakamura, T. et al. (1997). Role of hepatocyte growth factor in endothelial regulati[illegible] prevention of high D-glucose-induced endothelial [illegible] death b[illegible] prostaglandins and phosphodiesterase type 3 inhibit[illegible] *Diab[illegible]logia* **40**, 1053-1061.

Nakagami, H., Morishita, R., Yamamoto, K., Yo[illegible]mura, S. I[illegible]aniyama, Y., Aoki, M., Matsubara, H., Kim, S., Kan[illegible] Y. an[illegible] (2001). Phosphorylation of p38 mit[illegible]n-act[illegible]d [illegible]rotein kinase downstream of bax-caspase-3 pathway [illegible]s to cell d[illegible] induced by high D-glucose in human endothelial cell[illegible] *abetes* **50**, 147[illegible]81.

Nishikawa, T., Edelstein, D., Du, [illegible] Yamagishi, S., M[illegible]umura, T., Kaneda, Y., Yorek, M. A., Be[illegible], D., O[illegible] P. J., Hammes, H. P. et al. (2000). Normalizing mitochondrial superox[illegible] production blocks three pathways of hyperglycaem[illegible] damage. *Nature* **4[illegible]** 787-790.

Obrosova, I. G., Minche[illegible], A. G., Marinescu, V., [illegible]hallah, L., Kennedy, A., Stockert, C. M., [illegible]ank, R. N. [illegible]nd Stevens, M. J. (2001). Antioxidants attenuate early up[illegible]ulation of [illegible]al vascular endothelial growth factor in streptozotocin-diab[illegible]rats. *[illegible]betologia* **44**, 1102-1110.

Obrosova, I. G., Minch[illegible]. G., Vasup[illegible]m, R., White, L., Abatan, O. I., Kume[illegible] K., Fr[illegible] R. N. [illegible] Stevens, M. J. (2003). Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. *Diabetes* **52**, 864-871.

Quagliaro, L., Piconi, L., Assaloni, R., Martinelli, L., Motz, E. and Ceriello, A. (2003). Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes* **52**, 2795-2804.

Salgo, M. G., Squadrito, G. L. and Pryor, W. A. (1995). Peroxynitrite causes apoptosis in rat thymocytes. *Biochem. Biophys. Res. Commun.* **215**, 1111-1118.

Schroeder, P., Klotz, L. O., Buchczyk, D. P., Sad[illegible] C. D., S[illegible]ve, T. and Sies, H. (2001). Epicatechin selectively [illegible]events nitrati[illegible]but not oxidation reactions of peroxynitrite. *Bioc[illegible]. Biophys. Res. [illegible]mmun.* **285**, 782-787.

Schroeder, P., Klotz, L.-O. and Sies, [illegible] (2003). [illegible]phiphilic pro[illegible]ties of (–)-epicatechin and their signifi[illegible]ce for protec[illegible] of ce[illegible] against peroxynitrite. *Biochem. Biophy[illegible]es. Commun.* **307**, [illegible]73.

Shaw, S., Wang, X., Redd, H., [illegible]xander, G. [illegible], Isales, C. [illegible]nd Marrero, M. B. (2003). High glucose [illegible]ments th[illegible]giotensin II-induced activation of JAK2 in vascular sm[illegible]th m[illegible]s via the po[illegible]l pathway. *J. Biol. Chem.* **278**, 30634-3[illegible]1.

Suarez-Pinzon, W. [illegible] Szabo, C. and [illegible]inovit[illegible] A. (1997). Development of autoimmune [illegible] in NOD mice [illegible]ated with the formation of peroxynitrite [illegible] pancre[illegible] islet beta-cells. *[illegible]abetes* **46**, 907-911.

Suhara, T., Mano, T., [illegible]liveira, B. E. and Walsh, K. (2001). Phosphatidylinositol 3-kina[illegible] Akt signaling controls endothelial cell sen[illegible]ity [illegible] Fas-mediated ap[illegible]osis via regulation of FLICE-inhibitory p[illegible]ein (FLIP). *Circ. Res.* **89**, 13-19.

Tu[illegible]o, I. V., Marcon[illegible]es, S. and Murad, F. (2001). Diabetes-associated [illegible]ration of tyrosin[illegible] and inactivation of succinyl-CoA:3-oxoacid CoA-[illegible]nsferase. *Am. J. P[illegible]siol. Heart Circ. Physiol.* **281**, H2289-H2294.

van [illegible] Vliet, A., Hri[illegible]va, M., Cross, C. E., Eiserich, J. P. and Goldkorn, T. ([illegible]). Perox[illegible]ite induces covalent dimerization of epidermal growth factor re[illegible]n A431 epidermoid carcinoma cells. *J. Biol. Chem.* **273**, 31860-31866.

[illegible] B., Gallacher, B., Patel, H. and Orme, C. (1997). Glucose-induced protein[illegible]inase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. *Diabetes* **46**, 1497-1503.

Yue, T. L., Ni, J., Romanic, A. M., Gu, J. L., Keller, P., Wang, C., Kumar, S., Yu, G. L., Hart, T. K., Wang, X. et al. (1999). TL1, a novel tumor necrosis factor-like cytokine, induces apoptosis in endothelial cells. Involvement of activation of stress protein kinases (stress-activated protein kinase and p38 mitogen-activated protein kinase) and caspase-3-like protease. *J. Biol. Chem.* **274**, 1479-1486.

Zou, M. H., Shi, C. and Cohen, R. A. (2002). High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes* **51**, 198-203.

Zou, M.-H., Hou, X.-Y., Shi, C.-M., Kirkpatick, S., Liu, F., Goldman, M. H. and Cohen, R. A. (2003). Activation of 5′-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells: role of peroxynitrite. *J. Biol. Chem.* **278**, 34003-34010.