**NADPH Oxidase–Mediated ROS Production Determines Insulin’s Action on the Retinal Microvasculature**

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**PURPOSE.** To determine whether insulin induces nitric oxide (NO) formation in retinal microvessels and to examine the effects of high glucose on the formation of NO.

**METHODS.** Freshly isolated rat retinal microvessels were incubated in normal (5.5 mM) or high (20 mM) glucose with or without insulin (100 nM). The levels of insulin-induced NO and reactive oxygen species (ROS) in the retinal microvessels were determined semiquantitatively using fluorescent probes, 4,5-diaminofluorescein diacetate, and hydroethidine, respectively, and a laser scanning confocal microscope. The insulin-induced changes of NO in rat retinal endothelial cells and pericytes cultured at different glucose concentrations (5.5 and 25 mM) were determined using flow cytometry. Nitric oxide synthase (NOS) protein levels were determined by Western blot analysis; intracellular levels of ROS were determined using fluorescence-activated cell sorting (FACS) analysis of ethidium fluorescence; and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase RNA expression was quantified using real-time PCR.

**RESULTS.** Exposure of microvessels to insulin under normal glucose conditions led to a significant increase in NO levels; however, this increase was significantly suppressed when the microvessels were incubated under high glucose conditions. Intracellular levels of ROS were significantly increased in both retinal microvessels and cultured microvascular cells under high glucose conditions. The expression of NOS and NADPH oxidase were significantly increased in endothelial cells and pericytes under high glucose conditions.

**CONCLUSIONS.** The increased formation of NO by insulin and its suppression by high glucose conditions suggests that ROS production mediated by NADPH oxidase is important by insulin's effect on the retinal microvasculature.

Keywords: insulin, nitric oxide, ROS, high glucose, retinal microvessel

**Nitric oxide** (NO) is an endothelium-derived vasodilator, which plays a role in the autoregulation of ocular circulation. Insulin stimulates the formation of NO and increases ocular blood flow. Both insulin and NO are involved in the pathogenesis of diabetic retinopathy. Insulin treatment is related to the severity of diabetic retinopathy, and care must be taken because insulin therapy often causes a temporary worsening of the diabetic retinopathy. Reactive oxygen species (ROS), including superoxides (O₂⁻), are produced endogenously by endothelial cells in response to cytokines, growth factors, and/or shear stress. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the primary source of ROS in the retinal vasculature and diabetes can lead to increased ROS production through an upregulation of NADPH oxidase. Although redox signaling is required for the physiological regulation of blood flow, overproduction of ROS causes endothelial dysfunction through the quenching of local NO levels.

Therefore, we hypothesized that the devastating effects of insulin-induced NO on the retinal vasculature may be affected by the redox state of the retina. To test this hypothesis and determine whether insulin would induce NO formation, we used a laser scanning confocal microscope to collect time-lapse recordings of freshly isolated rat retinal microvessels. We showed that insulin exposure significantly increased NO formation in isolated retinal vascular cells under normal (5.5 mM) glucose conditions, while insulin exposure decreased NO formation under high (25 mM) glucose conditions. This paradoxical effect of insulin exposure was probably the result of increased ROS protein levels and increased ROS production following exposure to high glucose conditions.

**MATERIALS AND METHODS**

**Animals**

Twenty-five, 9-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). They were maintained on a 12-hour light/dark cycle and received food and water ad libitum. All rats used in these experiments were aged 9 to 16 weeks.
The experimental procedures used on the animals conformed to the guidelines outlined in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Osaka Medical College Committee on the Use and Care of Animals (Approval number: 23088).

**Chemicals**

All chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless specified.

**Isolation of Microvessels**

Retinal microvessels were isolated using the “tissue-print” method14 with some modifications. Briefly, retinas from euthanatized rats were rapidly removed and incubated in 2.5 mL Earle’s balanced salt solution (Life Technologies, Grand Island, NY, USA) supplemented with 0.5 mM EDTA, 1.5 mM CaCl2, 1 mM MgSO4, 20 mM glucose, 26 mM sodium bicarbonate, 15 U papain (Worthington Biochemicals, Freehold, NJ, USA), 0.04% DNase, and 2 mM cysteine for 30 minutes at 30°C. The medium was bubbled with 95% O2 to 5% CO2 for oxygenation and maintenance of the pH levels. The retinas were then transferred to solution A, which consisted of 140 mM NaCl, 3 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 10 mM Na-HEPES, 15 mM mannitol, and 5 mM glucose at pH 7.4, and an osmolarity adjusted to 310 mosmol.14 The retinas were then cut into four quadrants and each retinal quadrant was placed vitreous surface up in a glass-bottomed chamber containing 1 mL of solution AA. The coverslip was then positioned over the retina, and the tissue was gently sandwiched between the bottom of the chamber and the coverslip. When the coverslip was removed, complexes of microvessels adhered to the coverslip. We confirmed retinal vessels were not damaged by this procedure, as the cell viability of retinal microvessels assessed by trypan blue dye exclusion was 97.74% (n = 5) just after the isolation. Experiments were performed within 3 hours following the retinal vessel isolations, and time-lapse recordings of 2 to 3 microvessels/rat were monitored using a laser scanning confocal microscope.

Because the properties of pericytes located in the proximal and distal portions of the retinal microvasculature differ,15 we assessed the proximal portion of the pericyte-containing retinal microvessels, which were located distal to the smooth muscle-encircled arterioles and proximal to the capillaries (Fig. 1A). The density of pericytes in the proximal portion was >4/100 μm segment and the proximal portion of retinal microvessels could be distinguished from the distal part by their density.15 The mean ± standard deviation of the pericytes in the retinal vessels of our preparation was 4.4 ± 0.8/100 μm segment.

**Detection of NO Production in Freshly Isolated Microvessels**

The concentration of NO present in the freshly isolated retinal microvessels was determined by measuring the intensity of 4,5-diaminofluorescein diacetate (DAF-2DA; Cayman Chemical, Ann Arbor, MI, USA). 4,5-Diaminofluorescein diacetate reacts with NO to yield the highly fluorescent triazolofluorescein (DAF-2T), which can be quantified by measuring fluorescent intensities at 485 nm excitation and 538 nm emission wavelengths.16 Thus, we were able to semiquantitatively estimate NO local concentrations in the isolated retinal microvessels at a detection level as low as 5 nM.16 Time-lapse photographs were taken to determine fluorescent levels, which represented the amount of NO production. Twenty minutes after DAF-2DA was added to solution A at 37°C, the excess dye and solution were rinsed out. The coverslip containing the retinal microvessels were immediately moved to a 35-mm glass-bottom dish filled with solution A and mounted on the stage of a laser scanning confocal microscope (LSM 510 META, Carl Zeiss Meditec, Jena, Germany). After confirming that the baseline levels of fluorescence were stable, insulin (100 nM) was added to the media and photographs were taken every 20 seconds for 30 minutes. This concentration was chosen because the insulin’s effect to dilate vessels is observed below 100 nM in human,2 and the basal plasma concentration of insulin ranged between 50 to 60 nM in healthy and 100 nM in type 2 diabetes mellitus subjects.17 Microvessels under high glucose conditions were incubated for 60 minutes in a high glucose medium consisting of 115 mM NaCl, 3.0 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 10 mM Na-HEPES, 32 mM mannitol, 20 mM glucose at pH 7.4, and an osmolarity adjusted to 310 mosmol. This glucose concentration (20 mM) was determined by our previous findings that injection of 20 mM glucose increased ocular circulation.3 In addition, 20 mM glucose is reported to increase ROS production in retinal endothelial cells.18 These same vessels were then incubated for another 20 minutes following the addition of DAF-2DA at 37°C.

Nitric oxide levels were measured using >20 objective at 485 nm excitation and 505- to 550-nm emission wavelengths. The mean fluorescent intensity in each field (458 μm2) was serially measured using the embedded software (Zeiss LSM5; Carl Zeiss Meditec). The fluorescent intensity was expressed in arbitrary intensity units (AIUs). During the experiments, care was taken that the laser intensity, diameter of the diaphragm, photomultiplier sensitivity, and offset were constant for every set of measurements. Alterations in the NO production were expressed relative to the baseline levels. The means of the relative changes were calculated for each vessel, and then averaged for each experimental group.

**Detection of Intracellular Superoxide**

The level of superoxide in the isolated retinal microvessels was measured using hydroethidine, a fluorogenic probe. Hydroethidine is oxidized by superoxide to form ethidium, a fluorescent product, which is then retained intracellularly allowing a semiquantitative estimation of intracellular superoxide levels.19,20 Microvessels were incubated for 60 minutes in a high glucose media consisting of 115 mM NaCl, 3.0 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2, 10 mM Na-HEPES, 32 mM mannitol, 20 mM glucose at pH 7.4 and an osmolarity adjusted to 310 mosmol. These same vessels were then incubated for another 30 minutes following the addition of hydroethidine (1 μg/ml; 3.2 μM) at 37°C. After the addition of hydroethidine, microvessels were fixed for 15 minutes in the dark at room temperature using 4% paraformaldehyde in PBS. After a gentle wash with PBS, the coverslips containing the isolated microvessels were moved to a 35-mm glass-bottom plate filled with PBS and mounted on the stage of a confocal microscope (LSM 510 Meta; Carl Zeiss Meditec). The superoxide levels within the retinal microvessels were measured semiquantitatively with a >20 objective at 488-nm excitation and 590- to 610-nm emission wavelengths.20 The mean fluorescent intensity in each field (458 μm2) was measured and expressed in AUls.

**Cell Cultures**

A rat retinal capillary endothelial cell line, TR-iBRB2, and a rat retinal pericyte cell line, TR-rPCT, were obtained from Fact, Inc. (Sendai, Japan).21,22 These cell lines were collected from transgenic rats carrying a temperature-sensitive SV40 large T-
The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 33°C in a humidified atmosphere of 5% CO₂/air. Before reaching confluence, the media was changed to the control media (5.5 mM glucose in DMEM) or the high glucose media (25 mM glucose in DMEM, D6429), and the temperature was raised to 37°C in order to arrest the proliferation by reducing the expression of the large T-antigen. We then cultured the TR-iBRB2 and TR-rPCT cells in control or high glucose mediums lacking 10% FBS and serum-deprived overnight. Detailed procedures and treatment protocols for each experiment are described later.

Flow Cytometry (FACS) Analyses of NO and Superoxide Formation in Endothelial Cells and Pericytes

The formations of NO and superoxide were determined in the TR-iBRB2 and TR-rPCT cells using flow cytometry (EC800 Analyzer; Sony Biotechnology, Inc., Tokyo, Japan). Cells were harvested via trypsinization and centrifuged at 800g for 5 minutes. After washing with PBS, the cells were resuspended in phenol red-free DMEM in the presence or absence of insulin (100 nM) and incubated for 30 minutes at 37°C. Cell densities were adjusted to 2.0 × 10⁵ cells/mL. After incubation
cells via trypsinization, the cells were resuspended in phenol
wavelengths. This assay was performed in order to determine
fluoride hydrochloride (0.5 mM), aprotinin (0.15
lysed with a cell lysis buffer containing benzenesulfonyl
they were washed twice in PBS, harvested by scraping, and
Protein Levels of NOS by Western Blot

with insulin, the cells were incubated with DAF-2DA (5 µM)
for another 20 minutes at 37°C.

The intracellular levels of NO were determined using flow
cytometry (Sony Biotechnology, Inc.) at 488-nm excitation and
FITC (515–545 nm) emission wavelengths. The acquisition and
analysis software on the flow cytometer (Sony Biotechnology,
Inc.) was used to acquire and quantify the fluorescent
intensities. This assay was performed in order to determine
whether the insulin-induced NO formation was suppressed
under high glucose conditions.

The superoxide levels in the TR-iBRB2 and TR-rPCT cells
were measured using hydroethidine. After harvesting the
cells by trypsinization, the cells were resuspended in phenol
red-free DMEM at a density of 2.0 × 10⁵ cells/mL. The
intracellular levels of superoxide were measured semiquanti-
tatively at 488-nm excitation and 590- to 610-nm emission
wavelengths. This assay was performed in order to determine
the levels of superoxide generation by the cultured vascular
cells in control and high glucose mediums.

Protein Levels of NOS by Western Blot

We cultured the TR-iBRB2 and TR-rPCT cells in either control
or high glucose mediums for 2 days. After serum deprivation,
they were washed twice in PBS, harvested by scraping, and
lysed with a cell lysis buffer containing benzenesulfonyl
fluoride hydrochloride (0.5 mM), aprotinin (0.15 µM), leupep-
tin (1 µM), EDTA (20 mM), 0.1% SDS, 1.0% Nonidet P-40, 5.0%
sodium deoxycholate, Tris-HCl (50 mM, pH 7.6), and NaCl (150
mM). The lysed cell suspension was centrifuged at 10,000g for
15 minutes. The total protein concentration of the resulting
supernatant was determined using the Lowry method (DC
Protein Assay Reagent, Bio-Rad, Hercules, CA, USA).
Samples were separated on a 7.5% SDS-polyacrylamide gel and blotted
onto PVDF membranes. The membranes were then blocked
with 5% skim milk in Tris-buffered saline (pH 7.4) with 0.1%
Tween 20 (TBS-T) followed by an overnight incubation at 4°C
with each rabbit polyclonal antibody (1:1000) against NOS2
(M-19) Santa Cruz sc-650 (also designated iNOS) or NOS3 (C-
20) sc-654 (also designated eNOS; Santa Cruz, Dallas, TX, USA).
Tubulin (α-tubulin, 1:1000; Merck Millipore, CP06) was used as
an internal control. The membranes were washed three times
in TBS-T followed by incubation with a peroxidase-conjugated
goat anti-rabbit IgG (1:2500; Promega, Madison, WI, USA)
secondary antibody for 2 hours at 37°C. The protein bands
were visualized following the addition of an ECL plus Western
blotting detection system (GE Healthcare, Amersham, UK); exposure to film. Protein band densities were measured with a
luminescent image analyzer (LAS-3000, Fujifilm, Tokyo, Japan);
relative protein levels were quantified using the embedded
software (Multi Gauge version 3.0) and standardized according
to α-tubulin protein levels.

Real-Time PCR of NADPH Oxidase

Changes in NADPH oxidase (Nox) mRNA expression within
retinal vascular cells cultured in control or high glucose
mediums were determined using real-time PCR. The retinal
endothelial cells and pericytes were cultured in control or high
glucose mediums as previously described, washed in PBS, and
harvested by scraping in buffer (RIT plus; Qiagen, Valencia, CA,
USA). Total RNA was extracted using an RNA extraction kit
(RNeasy plus mini kit; Qiagen), and the RNA quality and
quantity was assessed using a spectrophotometer (BioSpectrom-
eter; Eppendorf, Hamburg, Germany). The purified RNA was
then reverse transcribed with commercial RT-PCR kits according
to the manufacturer’s instructions (PrimeScript RT reagent and
gDNA Eraser; TaKaRa, Ohtsu, Shiga, Japan). Quantitative real-
time PCR analysis was performed using a real-time system
(Thermal Cycler Dice, TP870; TaKaRa); Premix Ex Taq (Perfect
Real Time; TaKaRa); and the TaqMan gene expression assays for
the targeted genes (Applied Biosystems, Foster City, CA, USA).
TaqMan gene assays for NADPH Rn00585380_m1 were used.
Amplicons were detected using the relevant probes tagged with a
MGB quencher and a FAM dye. All reactions were run with the
following cycling parameters: 30 seconds at 95°C followed by 40
cycles at 95°C for 5 seconds and 60°C for 30 seconds. A standard
curve of the cycle thresholds was established using serial
dilutions of the cDNA samples. The relative quantities of mRNAs
were calculated with 18s (Hs99999901_s1) serving as our
internal control.

Statistical Analyses

The means and standard errors of the means were calculated,
while a one-way ANOVA was performed. If a significant change
was detected, then a Scheffe’s test was done for statistical
comparisons between groups. Unless noted, the two-tailed
Student’s t-tests were used. The level of significance was set at
P < 0.05.

RESULTS

Effects of Insulin on DAF-2T Fluorescent
Intensities in Isolated Retinal Microvessels

Time-lapse fluorescence micrographs of freshly isolated retinal
microvessels after exposure to insulin are shown in Figure 1.
No apparent changes were observed in DAF-2T fluorescent
intensities in retinal microvessels incubated in control media
without the addition of insulin (Fig. 1A, control). Exposure of
the retinal microvessels to insulin in the control media
increased DAF-2T fluorescent intensities (Fig. 1B). On the
other hand, there was a slight decrease in DAF-2T fluorescent
intensities when the retinal microvessels were cultured in high
glucose media lacking insulin (Fig. 1C), while the addition of
insulin to the high glucose media further decreased the DAF-2T
fluorescent intensities (Fig. 1D). Quantification and statistical
validation of the insulin-induced increase in DAF-2T fluorescent
intensities relative to the control levels are shown in
Figure 1E. Exposure of the retinal microvessels to insulin in the
control media significantly increased DAF-2T fluorescent

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intensities \((P = 0.003\), Scheffe\), while DAF-2T intensities were significantly reduced in high glucose media with the addition of insulin \((P = 0.001\), Scheffe\).

**Effects of High Glucose on Hydroethidine Intensities in Isolated Retinal Microvessels**

The levels of superoxide within the isolated retinal microvessels were quantified by measuring ethidium intensities. These results are shown in Figure 2. Ethidium intensities within the retinal microvessels incubated in high glucose media \((25\, \text{mM} \text{ glucose})\) was significantly higher than in the retinal microvessels incubated in control media \((5.5\, \text{mM} \text{ glucose})\).

**FACS Analyses of Insulin-Induced DAF-2T Fluorescein Intensities**

Representative graphs showing the changes of insulin-induced NO formation in TR-iBRB2 cells are shown in Figure 3.

![Figure 3](http://arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934564/)

**FACS Analyses of Ethidium Fluorescent Intensities**

We examined the generation of ROS in cultured vascular cells adding hydroethidine and measuring ethidium fluorescence by FACS analysis. Representative changes in ethidium fluorescent intensities for endothelial cells and pericytes are shown in Figures 4A and 4B, respectively. Ethidium fluorescent intensities were plotted on the absorbssa, while the number of cells were plotted on the ordinate. Ethidium fluorescence was increased in both the TR-iBRB2 and TR-rPCT cells cultured under high glucose conditions when compared with cells cultured in control media. These findings indicate that superoxide anions were generated in the presence of high glucose.

**Changes of NOS Expression in High Glucose Media**

Protein levels of NOS in endothelial cells and pericytes cultured under control or high glucose conditions were determined by Western blot analysis and subsequent quantification (Fig. 5). The expression of iNOS and eNOS were significantly increased in both TR-iBRB2 and TR-rPCT cells cultured under high glucose conditions \((P < 0.05\), Student’s \(t\)-tests).

**Gene Expression of Nox4 in High Glucose Media**

Real-time PCR was performed for the NADPH oxidase subunit \(Nox4\). Expression of RNA in NAPDH oxidase subunit \(Nox4\) was significantly increased in both endothelial cells \((F6; P < 0.01\), Student’s \(t\)-test) and pericytes \((F6b; P < 0.01\), Student’s \(t\)-test) cultured under high glucose conditions.
compared with the expression of NADPH oxidase subunit Nox4 in cells grown under control conditions.

**DISCUSSION**

Our results showed that insulin increased NO formation in isolated retinal vessels exposed to control media consisting of physiological concentrations of glucose (5.5 mM), but decreased the formation of NO in high glucose media (20 mM glucose). Similar findings were obtained in cultured retinal vascular cells as determined by FACS analyses. Endothelial cells and pericytes cultured under high glucose culture conditions demonstrated an increase in the expression of NOS (eNOS and iNOS); however, the expression of superoxide and Nox4 was also increased.

Nitric oxide is closely associated with the regulation of retinal circulation by inhibiting the decrease of regional blood flow via NOS. Insulin dilates retinal vessels through the formation of NO, and diabetes is one of the factors associated with the resistance of blood vessels to insulin. In turn, this insulin resistance can cause endothelial damage and impair the formation of NO. Although our results were obtained from isolated retinal vessels incubated in high glucose media and not from vessels of diabetic animals, the high glucose media clearly suppressed the insulin-induced NO formation. Moreover, insulin reversibly decreased NO formation in endothelial cells cultured under high glucose conditions. It is unlikely that the opposite action of insulin was due to the downregulation of NOS because the expression of NOS (iNOS and eNOS) was significantly increased in both the endothelial cells and pericytes cultured in high glucose media. The upregulation

**FIGURE 5.** Protein levels of NOS by Western blot analyses.

**FIGURE 6.** Real-time PCR of NADPH oxidase subunit Nox4 in (A) endothelial cells and (B) pericytes.

* P<0.05 (t-test)

* P<0.01 (t-test)
of NOS expression under high glucose conditions is in agreement with a previous report that showed protein levels of all NOS isoforms were increased in the retinas of diabetic mice.7

Insulin-induced vasodilation is known to be impaired whereby superoxide reacted with NO and eliminated the action of NO-induced vasodilation.27–29 In some cases, NOS inhibition paradoxically promoted vasodilation, whereby excess amounts of superoxide caused the oxidation of NOS cofactors, tetrahydrobiopterin (BH4). This previously reported observation may account for our finding that insulin decreased the NO formation in endothelial cells cultured under high glucose conditions. To further support this observation, we demonstrated that high glucose conditions increased ethidium fluorescent intensities of isolated retinal vessels and cultured retinal endothelial cells and pericytes. We also found an upregulation of NADPH oxidase subunit Nox4 in retinal endothelial cells and pericytes cultured under high glucose conditions. Nicotinamide adenine dinucleotide phosphate oxidase is a primary source of superoxide anions in retinal microvessels,12 which are also the target of insulin.26 Thus, these findings suggest that high glucose conditions cause oxidative stress, while insulin enhances the formation of superoxide through the uncoupling of NOS and NADPH oxidase.

In patients, insulin is a risk factor for diabetic macular edema.4,6 In addition, some diabetic patients have a temporary worsening of diabetic retinopathy following insulin therapy. Our results support the idea that insulin may alter its effect on retinal vessels in a redox-state dependent manner. Thus, antioxidant therapy may alleviate any diabetes-induced vascular changes.30

There are some limitations of this study. First, one limitation of our study is that our results were obtained in vitro from freshly isolated retinal vessels of healthy rats, and cultured rat retinal microvascular cells. Thus, direct links of our findings to human diabetic retinopathy should be considered cautiously. It would be important to confirm the present results using diabetic animals further. Second, we performed various quantitative analyses by using fluorescent probes. However, influences of quenching or autofluorescence of the dye should also be considered on our results. In addition, we did not precisely determine the mechanisms of how insulin interacts with NOS and NADPH oxidase; therefore, these issues will need to be further investigated.

In conclusion, our findings show that insulin-induced NO formation in isolated retinal microvessels and retinal endothelial cells could be reversed under high glucose conditions, while insulin decreased NO formation under high glucose conditions where superoxide was in excess (Fig. 7). Therefore, ROS production of retinal microvasculature appears to be important for insulin’s vascular action on retinal circulation.

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References


FIGURE 7. A schematic diagram of biochemical changes related to the action of insulin in relation to NO and ROS in the retinal microvasculature. References are numbered at key mediators in this figure.
Insulin-Induced Nitric Oxide in Retinal Microvessels


