Role of NADPH oxidase and angiotensin II in diabetes-induced retinal leukostasis

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Abbreviations: AO: acridine orange; FITC: fluorescein isothiocyanate; RAS: renin-angiotensin system; SLO: scanning laser ophthalmoscope; VEGF: Vascular Endothelial Growth Factor;
Abstract

We studied whether angiotensin II via superoxide may contribute to retinal leukostasis and thus to the pathogenesis of retinopathies. We studied: 1. whether intravitreal (ivt) angiotensin II induces retinal leukostasis that is altered by antioxidants or by apocynin, a NAD(P)H oxidase inhibitor and 2. Whether retinal leukostasis induced by diabetes in rats is also altered by these treatments. Rats were injected ivt with angiotensin II (20 µg in 2 µl), and divided into three groups: 1. Untreated; 2. Treated with tempol (~70 mg/kg/day) and NAC (~1g/kg/day); 3. Treated with apocynin (~45 mg/kg/day), both in the drinking water. Rats with streptozotocin-induced diabetes were similarly treated. Leukostasis was evaluated 48 hr after angiotensin II or two weeks after diabetes induction. Angiotensin II increased retinal leukostasis from 0.3 ± 0.5 to 3.7 ± 0.4 leukocytes/mm² (p< 0.01) and these changes were markedly decreased by treatment with tempol+NAC or apocynin, and also by a blocking antibody against VEGF given ivt (p< 0.01). In addition, incubation of dihydroethidium-loaded retina sections with angiotensin II caused marked increase in superoxide formation. Compared with normal controls, retinal leukostasis in diabetic rats markedly increased from 0.2 ± 0.3 to 3.8 ± 0.1 leukocytes/mm² (p< 0.01). Diabetic retinal leukostasis was also decreased by treatment with tempol-NAC and normalized by apocynin. Thus increases in intravitreal angiotensin II can induce retinal leukostasis, that appears mediated via increasing superoxide generation by NAD(P)H oxidase, and by VEGF. The activity of NAD(P)H oxidase is required for leukostasis to occur in the diabetic retina.
Introduction

Diabetic retinopathy is a major cause of blindness (2;3) that is characterized by progressive alterations in the retinal microvasculature, manifested by areas of non-perfusion, increased permeability, leaky microaneurysms, and pathological angiogenesis. Two sets of observations suggest that the renin-angiotensin system (RAS) may contribute to the pathogenesis of diabetic retinopathy. First, the components of the RAS are present in the eye, and intraocular and serum levels of angiotensin II (Ang II), renin, and angiotensin-converting enzyme (ACE) reportedly correlate significantly with the severity of retinopathy (10;16-18;52;56). Second, ACE inhibitors (ACEi) reportedly arrest or delay progressive breakdown of the blood-retina barrier in normotensive insulin-dependent diabetic patients with nephropathy and have favorable effects on normotensive patients with insulin-dependent diabetic retinopathy (21). These finding suggest the possibility that Ang II acts as an endocrine/paracrine factor and, thereby, influences the development of diabetic retinopathy, as proposed by some investigators (59).

Recent evidence suggests that leukocytes may be involved in the genesis of diabetic retinopathy and this may be linked to the RAS. The onset of diabetes appears to be accompanied by marked retinal leukostasis and retinal capillary non-perfusion secondary to leukocyte-induced retinal capillary plugging (39;40;44). Two main receptors have been reported for Ang II, AT₁ and AT₂. Most of the known cardiovascular effects of Ang II are mediated by AT₁ (57), but in some cases AT₂ may also be involved (9). Mori et al (41) reported that an AT₁ antagonist decreased leukocyte entrapment within the retina of diabetic rats, and we showed that treatment with an ACEi (ramipril) or the angiotensin AT₁ antagonist losartan drastically reduced retinal leukostasis in rats with early diabetes (11), thus implicating the RAS in diabetic retinal leukostasis. Although Ang II can increase expression of leukocyte adhesion molecules and thereby induce leukocyte recruitment, leukostasis, and inflammation (42), to our knowledge this has not been shown in the retina.

NAD(P)H oxidase activity is increased in the retina of diabetic rats (20). Growing evidence suggests involvement of oxidative stress in the vascular
complications associated with diabetes, including retinopathy (27). It has been suggested that leukostasis may be associated with endothelial dysfunction and oxidative stress (1). Endothelial cells respond to Ang II with increases in superoxide formation, mediated by assembly and activation of NAD(P)H oxidases (54). Angiotensin-induced superoxide has been implicated as a key factor in a variety of situations characterized by vascular dysfunction (37). In this study we address the hypothesis that Ang II induces retinal leukostasis associated with activation of NAD(P)H oxidases. For this we assessed 1) whether injecting Ang II into the vitreous chamber of the eye would result in increased retinal leukostasis, and whether these effects are mediated by the AT_1 and/or AT_2 receptor; 2) whether these effects of Ang II could be altered by a) general antioxidants such as tempol (a superoxide-selective scavenger) and N-acetyl-cysteine (NAC), a glutathione-based antioxidant, and/or b) apocynin, an inhibitor of NAD(P)H oxidase; and finally, 3) whether the same antioxidant compounds would affect diabetes-induced retinal leukostasis.
Materials and Methods

Animals

Male Long-Evans rats (Charles River Laboratories, Wilmington, MA) weighing 200-225 g were used. All experiments were conducted in accord with the Institutional Animal Care and Use Committee (IACUC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light-dark cycle.

Induction of Diabetes

After fasting overnight, the experimental group received a single 65 mg/kg intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) in 10 mM sodium citrate buffer, pH 4.5. Rats in the control (non-diabetic or normal) group were injected with citrate buffer alone. Thereafter the rats underwent no further fasting.

To measure glucose levels, blood was obtained by pricking the tail vein-artery complex with a 23-gauge needle. Blood glucose was quantitated with an automated test strip employing the glucose oxidase method (Accu-Chek; Roche Diagnostics, Indianapolis, IN) as per the manufacturer’s instructions. Estimates were made by comparing readings to a standard curve (100-400 mg D-dextrose/dl). Differences as low as 10 mg/dl can reproducibly be detected with this method. In all rats, blood glucose was measured 72 hr after citrate or streptozotocin (STZ) treatment. Among the STZ-treated rats, only those with blood glucose concentrations greater than 250 mg/dl were considered diabetic and used for subsequent experiments. After screening, blood glucose was measured at 3, 7, and 12 days.

Ang II & Antioxidants: Rats were randomly assigned to one of four experimental groups, each comprising 6-8 animals:
Protocol 1. Objective: to determine whether intravitreal (ivt) injection of Ang II induces retinal leukostasis and, if so, whether these effects are mediated by AT\textsubscript{1} and/or AT\textsubscript{2} receptor.

1) Normal controls injected ivt with saline
2) Ang II (20 µg) given ivt
3) Treated with ivt Ang II and the Ang II AT\textsubscript{1} receptor antagonist losartan (20 µg).
4) Treated with ivt Ang II and the highly selective Ang II AT\textsubscript{2} receptor antagonist PD123319 (20 µg).

Ang II and the receptor antagonists were mixed and thus given simultaneously in 5 µl volume.

The ivt injection was performed by inserting a 27 or 30-gauge needle into the vitreous humor 1 mm lateral to the limbus. The tip of the needle was positioned over the optic disk, and 5 µl was slowly injected into the vitreous humor.

The dose of Ang II was determined after pilot experiments in which 1, 5 and 20 µg were injected ivt (n = 2 each) and the retinas analyzed for leukostasis using the LSO-acridine orange technique. Moderate leukostasis was observed with 5 µg/eye, but it was obvious with 20 µg/eye, which was used thereafter for all experiments. The dose of losartan was determined in pilot experiments showing that it inhibited more than 50% of the response to ivt Ang II (20 µg). PD123316 was then administered at the same dose.

Protocol II. Objective: to determine whether the retinal leukostasis induced by ivt Ang II was affected by inhibiting vascular endothelial growth factor (VEGF).

1. Controls injected with saline
2. Treated with ivt Ang II (20 µg)
3. Treated with ivt Ang II + SU5416 (10 µg)
4. Treated with ivt Ang II + VEGF neutralizing antibodies (100 ng)
Ang II and the VEGF inhibitors were mixed together and given simultaneously in 5 µl volume. Our doses of SU5416 and VEGF monoclonal antibodies (Chemicon, Temecula, CA) blocked more than 80% of the retinal leukostasis induced by ivt VEGF (100 ng). The rest of the protocol is identical to that described above.

Protocol III: Objective: to determine whether the effects of Ang II in retinal leukostasis are dependent on formation of reactive oxygen species (ROS).

1. Treated with ivt Ang II
2. Treated with ivt Ang II + tempol+ N-acetyl-cysteine (NAC).
3. Treated with ivt Ang II + apocynin

The antioxidants were given in the drinking water as described below.

Antioxidant treatment was begun 3 days before ivt injections, at a dose of ~3 mM/day tempol, a superoxide dismutase mimetic, ~1 g/kg/day N-acetyl-cysteine (NAC), a glutathione based antioxidant, or ~2 mM/day apocynin, a specific inhibitor of NAD(P)H oxidase assembly and activity. Treatments continued for 48 hrs after ivt injections, at which time animals were evaluated for retinal leukostasis.

**Quantitating Leukostasis by Laser Scanning Ophthalmoscopy**

Leukostasis was quantitated in the retina by the method pioneered by Ogura (46) and modified by Barouch et al. (5). With this technique, leukocytes are labeled *in vivo* by intravenously infusing the fluorescent agent, acridine orange (AO), which binds to nucleated cells (including leukocytes). At 14 days after beginning drug treatment, rats were anesthetized with 10 mg/kg xylazine (Phoenix Pharmaceutical, St. Joseph, MO) and 80 mg/kg ketamine (Parke-Davis, Morris Plains, NJ) given intramuscularly. Subsequently, polyethylene (PE-50) catheters filled with diluted heparin were surgically implanted in the right femoral vein and externalized subcutaneously.

To prepare the fluorescence-containing infusate, AO (Sigma) was dissolved in sterile saline (1.0 mg/ml) and further sterilized by microfiltration. After 24 hr of recovery from surgery, each rat was anesthetized as before, the right pupil was
dilated with 1% tropicamide (Alcon, Humancao, Puerto Rico), and a contact lens (Unicon, Osaka, Japan) was placed in the eye. A focused image of the peripapillary fundus of the right eye was obtained with a scanning laser ophthalmoscope (SLO; Rodenstock, Ottobrunn-Rienerling, Germany) using an argon blue laser as the illumination source and a standard fluorescein angiography filter and a 40° field setting. Following this, the AO solution was infused through the femoral vein catheter at a rate of 1 ml/min until a dose of 3 mg/kg was delivered to each rat. Leukocyte labeling by AO within the retinal microcirculation could be observed immediately thereafter via the SLO. After additional 20 min, SLO images of the fundus were recorded for 2 min and stored. FITC-dextran (MW = 2000 kDa; made up as a 20:1 dilution of a 5% solution) was then immediately injected as an iv bolus into the femoral vein to confirm that the leukocytes identified by AO remained static. These large dextran molecules are distributed in the plasma, do not bind to blood cells, and do not cross the normal or intact blood-retinal barrier. Therefore, when viewed through the SLO, the fluorescence of this FITC-dextran, demarcates the portion of the retinal microcirculation that is perfused by plasma. From 1-3 min after injection, a set of FITC-dextran images were obtained with the SLO as described above for AO labeling of leukocytes. The vast majority (>90%) of the leukocytes were found to localize intra-vascularly. A few (10%) appeared to occlude blood flow, but this was difficult to ascertain with accuracy. These results are not shown because the FITC-dextran studies did not provide qualitatively better data than AO alone.

**Image Analysis of SLO Data**

The video recordings were analyzed on a computer equipped with a video digitizer that processes video images in real time. Leukostasis was determined from the 20-22min AO data. An AO-labeled leukocyte was considered static if its position did not change for the 2 min observation period. The density of static leukocytes was calculated by manually counting the number of such spots of AO fluorescence in the 640 x 480 pixel area as originally described by Nishiwaki (32) and reviewed by Ogura (24). Using a calibration factor obtained by measuring
optic disk diameter, we obtained the relationship between pixels and µM as observed in the SLO (0.41 pixels/µM). The data were converted to number of static leukocytes per mm² and will be presented as such.

Over the 22 min of the SLO-AO protocols, the number of labeled, static leukocytes within the retina would be expected to be few – perhaps no more than 10 per SLO field – and to remain within the microvessels of the retina. The microvascular networks are not apparent from the AO images. However, FITC-dextran fills the plasma portion of the retinal microvascular system. Accordingly, we used the FITC-dextran images to illustrate this network of small arterioles, capillaries, and small venules and to visually correlate with the sites of AO-leukostasis.

Concanavalin A Lectin and CD45-Antibody Co-labeling of Leukocytes

A second approach to quantitating leukostasis involved fixed, flat-mounted retinas and immunoreactive labeling of leukocytes with FITC-concanavalin A-lectin and CD45-antibody. These experiments were done in separate groups of Ang II-treated and diabetic rats (n = 4/treatment group). Concanavalin A lectin labels both adherent leukocytes and microvascular endothelial cells, CD45 is a hematopoietic-cell-specific tyrosine phosphatase that is a common leukocyte marker (53). After perfusing, fixing, and staining the retina as described below, a leukocyte was considered adherent or static when the fluorescence of both labels was coincident, i.e., localized to the same spot.

Lectin labeling was performed as described by Ishida et al. (32). The animals were anesthetized as described above. The chest cavity was opened and a 14-gauge infusion cannula inserted into the left ventricle. Blood and perfusate drainage from the perfused circulatory system was achieved by placing an 18-gauge needle in the right atrium. Approximately 100 ml phosphate-buffered saline (PBS) was infused over 2 min to wash out most, if not all, of the blood from the body. In particular, this was done to remove all blood cells that did not adhere to the retinal microvasculature. Immediately afterward, fluorescein-isothiocyanate (FITC)-coupled concanavalin A lectin in PBS (pH = 7.4;
concentration = 60 µg/ml; volume infused = 16-18 ml; Vector Labs, Burlingame, CA) was infused into the heart and through the entire vascular system. This was followed by removal of residual unbound lectin by perfusion with 20-25 ml PBS. Retinas were removed and fixed by adding 1% formaldehyde to cover the flat mounts. After 60 min, the flat mounts were imaged using a fluorescence microscope (Leica DEM IRB, Bannockburn, IL). The whole retina was scanned for adhering leukocytes. Because of the infusions of PBS and fixative, most or all of the unattached leukocytes were likely to be washed out of the system; consequently, it was assumed that all retained leukocytes were adherent at the time of perfusion and fixation. The separate images of concanavalin-A and CD45 associated fluorescence were electronically merged by image analysis software (NIH Image, Scion, Frederick, MA). The spots of coincident fluorescence in the whole retina were manually counted using a 20x objective. Results are expressed as number of leukocytes\ per retina.

**CD45 Immunofluorescence**

To confirm the identity of the adherent cells within the vasculature, CD45 immunofluorescence was performed in the control Ang II-treated and diabetic retinas. Arrested leukocytes were labeled with fluorescein isothiocyanate-linked concanavalin A as described above. The flat-mounted retinas were permeabilized with 0.5% Triton X 100 (Sigma) in PBS for 24 hr, and nonspecific binding was blocked with 5% normal goat serum. The retinas were then incubated with a mouse anti-rat CD45 antibody (clone OX-1, 1:500; BD Pharmingen, San Diego, CA) overnight at 4°C, followed by incubation with a Texas red–conjugated goat antibody against mouse immunoglobulins (1:200; Jackson ImmunoResearch, West Grove, PA). The retina flat mounts were prepared with a mounting medium for fluorescence (Southern Biotechnology, Irvine, CA) and imaged with a Leica DM IRB epi-fluorescence microscope.
Dihydroethidium (DHE) Imaging

Production of superoxide in retinal sections was assayed using dihydroethidium (DHE), a fluorescent dye that in the presence of superoxide is oxidized to oxyethidium, which binds to nuclear DNA and fluoresces red. Enucleated normal rat eyes were flash frozen in liquid nitrogen and allowed to harden. Serial 20 µm sections were incubated in sterile PBS, 0.1% D-glucose, L-arginine (50 µM) and NAD(P)H (100 µM). The sections were incubated with Ang II (1 µM) alone, Ang II and apocynin (100 µM), or vehicle (0.1% EtOH). Thirty minutes later, the medium was replaced by fresh solutions of these same compounds and DHE (2 µM) and incubated for additional 30 min, followed by fluorescence microscopy and image capture. Fluorescence intensity was quantitated using SigmaScan Pro 5 software for image analysis.

VEGF Immunoreactivity

Frozen sections (20 µm) were obtained from the ocular globes of rats treated ivt with either saline or Ang II. The sections were fixed by incubating with 4% paraformaldehyde for 30 min and washed 3 times with 0.5% Triton X 100. This was followed by cold methanol-3% H₂O₂ and additional washes with 0.5% Triton X 100. After incubation for 30 min with 20% goat serum, a 1:50 dilution of the VEGF monoclonal antibody (Chemicon, Temecula, CA) was added and the samples were incubated overnight at 4°C. After washing with PBS, we added 1:200 dilution of Texas-red dye-conjugated AffiniPure goat anti-mouse IgG for 30 min. The sections were washed first with PBS, then with 70% ethanol for 2 min, and with absolute ethanol for 2 min. Finally, xylene was added for 2 min, and the sections were dried and mounted for fluorescence microscopy.

Statistics

All results are expressed as mean ± SEM. The data were compared by analysis of variance, testing post hoc comparisons using Fisher’s protected least significant difference procedure.
Results

Intravitreal administration of Ang II induced marked retinal leukostasis, as clearly observed using the SLO-AO technique. The density of static leukocytes was more than 4 times greater in the eyes injected with Ang II than in the eyes injected with saline (Fig 1). This effect was statistically significant ($p < 0.01$). Furthermore, we found that co-treatment with the AT$_1$ antagonist losartan significantly reduced Ang II-induced retinal leukostasis (Fig 1). Treatment with the AT$_2$ antagonist PD123319 did not significantly reduced the leukostasis induced by Ang II (Fig 1).

In diabetes, VEGF mediates diabetic retinal leukostasis (32) and Ang II is capable of stimulating VEGF in some cells (13;50). We tested whether Ang II-induced retinal leukostasis would be affected by inhibitors of VEGF. Both SU5416 and VEGF neutralizing antibodies significantly reduced the Ang II-induced retinal leukostasis (Fig 2). In addition, we documented moderate increases in VEGF immunoreactivity, particularly in the ganglion cell layer, which contacts the vitreous humor (Fig 3).

Based on the premise that Ang II exerts effects on vascular cells in part by inducing the formation of reactive oxygen species (ROS), we examined whether antioxidants would affect Ang II-induced retinal leukostasis. Rats were given either tempol/NAC or apocynin in the drinking water, and Ang II was administered ivt. Treatment with tempol/NAC markedly reduced retinal leukostasis, while apocynin abolished it altogether (Fig 4). To confirm these results, we assessed retinal leukostasis by the FITC concanavalin A technique. Identity of cellular elements detected by the concanavalin A label was reinforced by confirming co-localization with immunoreactive CD45. The results were very similar to those obtained with SLO-AO. Ang II-induced retinal leukostasis was markedly reduced by tempol/NAC and abolished by apocynin (Fig 5).

Since the data suggested that Ang II induces oxidative stress in the retina that appears to be mediated by activation of NADP(H) oxidase, we attempted to document this by loading sections from snap frozen eyes of normal rats with
DHE and studying whether adding Ang II increased fluorescence, and whether this was reduced by apocynin. Ang II caused an increase in fluorescence intensity in the cells from the retinal ganglion layer, as well as the inner and outer nuclear layers, that were abolished by apocynin (Fig 6).

We then studied whether retinal leukostasis in streptozotocin-induced diabetic rats was also affected by treatment with tempol/NAC or apocynin. Two weeks after induction of diabetes, retinal leukostasis was assessed in untreated diabetic rats and in diabetic rats treated with either tempol/NAC or apocynin, using SLO-AO in some animals and FITC-concanavalin A in others. The diabetic rats exhibited marked retinal leukostasis (Fig 7) similar to the Ang II induced elevation in leukostasis observed in non-diabetic rats (Fig 1). As was the case in the non-diabetic rats this effect was greatly reduced by both tempol/NAC and apocynin (Fig 7). These results were documented by both SLO-AO (Fig 7) and concanavalin A (Fig 8).
Discussion

All components of the RAS including the angiotensin II type 1 and type 2 receptors, have been identified in the retina of humans and rodents. There is evidence from both clinical and experimental models of diabetic retinopathy and hypoxia-induced retinal angiogenesis that the RAS is up-regulated. Evidence to date indicates that blockade of the RAS can confer retino-protection in experimental models of diabetic and ischemic retinopathy (15;60).

It has been reported that the retinal leukostasis observed in early diabetic rats is markedly reduced by an ACE inhibitor or by losartan, an angiotensin AT\textsubscript{1} antagonist (11;41), suggesting that Ang II is involved in this process. However, it was not known whether increases in the ocular concentration of Ang II would result in retinal leukostasis. We assessed the effects of Ang II on retinal leukostasis by two methods: 1) SLO-AO (5;43;46), and as a second approach for studying leukostasis and confirming the SLO-AO findings, 2) staining retinal flat mounts with FITC-concanavalin A. The SLO-AO method allows us to identify nucleated cells, seen as fluorescent dots in microvessels in the peri-fundal area. Cells arrested in larger vessels are not clearly visible because of the intense background fluorescence, while areas farther away from the optic disc are also difficult to scan. AO will also label cells of the vascular wall. An advantage of the SLO-AO method is that the observations are done \textit{in vivo} and in real time. Under these conditions, most if not all of the arrested leukocytes appeared to be trapped within the capillaries and small venules (Fig. 1). When AO was followed by FITC-dextran, the lumina of the vessels became filled with fluorescence, and around 10\% of the fluorescent dots appear to induce discontinuity in the FITC-dextran fluorescence. This may reflect trapped leukocytes that occlude the microvascular circulation; but it is uncertain whether this reflects alterations in vascular patency. SLO imaging provides only a brief snapshot of retinal leukocytic arrest (a few min at best). Assuming that some capillaries were indeed occluded, it cannot be determined if this is a short-lived or long-term event, and in any case it may not occur that frequently.
Our second approach, staining leukocytes with FITC-concanavalin A, was performed on perfused and fixed retinal flat mounts. In some retinas the identity of the cells counted as white cells was confirmed by co-localization with CD45 immunoreactivity, a pan-leukocyte marker. The concanavalin-A+CD-45 approach involved prolonged flushing of the vascular system with saline. Most of the static leukocytes were clearly visible in veins, and these were the ones we counted. Perhaps the prolonged saline perfusion dislodged leukocytes that were not firmly attached to the endothelial cells in other parts of the vasculature.

With both approaches, the retinas of rats treated with ivt Ang II had significantly more trapped leukocytes than the saline-treated controls. Thus Ang II promotes inflammation in the retina, as observed in other tissues (12;51). In most reports dealing with the pro-inflammatory effects of Ang II, the effects were found to be mediated by the AT₁ receptor (12), although in some instances the AT₂ receptor has also been implicated (22). Consistent with most studies, we also found that Ang II-induced retinal leukostasis was mediated by activation of the AT₁ receptor.

There were differences in the number of adhered leukocytes in the control groups from the different protocols. It could be that this was simply due to the fact that experiments were done at different times and with different rats. However the rats of the protocols involving studying the effects of ivt Ang II received ivt saline in the contralateral eye. The eyes in which we studied the effects of treatments on the diabetic rats were not manipulated in any way. It may be that the ivt procedure by itself has a small pro-inflammatory effect.

Diseases such as diabetes are associated with changes in vascular function and structure, including endothelial dysfunction and vascular remodeling. Several stimuli, including vasoactive agents (of which Ang II appears to be one of the most important) can influence cellular changes. Ang II mediates many of its pleiotropic vascular effects through NAD(P)H oxidase-derived ROS. To determine whether ROS are involved in Ang II-induced retinal leukostasis, we pretreated the rats with a combination of the antioxidants tempol, a superoxide scavenger, and N-acetylcysteine, a glutathione-conserving agent. The doses
used were selected because they have been utilized by others (29;31;58). This treatment caused marked reduction of the retinal leukostasis induced by ivt Ang II, suggesting that it was mediated by ROS. To establish whether these ROS were the product of superoxide formation induced by activation of NADP(H) oxidase, the diabetic rats were treated systemically with the NADP(H) oxidase inhibitor apocynin (7;33), which blocks NAD(P)H oxidase activity by interfering assembly of the cytosolic NAD(P)H oxidase components p40^phox, p47^phox, p67^phox with the membranous components gp91^phox and p22^phox and therefore impairs NADP(H) oxidase activity (14). Apocynin abolished the retinal leukostasis induced by ivt Ang II. These data clearly implicate superoxide anion produced by NADP(H) oxidase as a necessary component for ivt Ang II to induce leukocyte accumulation in the retina.

The control eyes that received ivt saline had somehow higher arrested leukocytes that the control eyes that had not been given any treatment. Perhaps the ivt injection caused by itself a slight inflammatory response reflected by a few arrested white blood cells.

There was no qualitative difference in the data obtained using SLO-AO and concanavalin A. Although these techniques measure different events, they appear to be interchangeable. With both techniques, Ang II increased retinal leukostasis and antioxidants/apocynin inhibited this increase.

Increased superoxide has been reported to stimulate VEGF synthesis and activity (48;54). VEGF administered ivt causes retinal leukostasis and plays a role in the retinal leukostasis of streptozotocin-induced diabetes (32). Ang II induces superoxide formation by activating NAD(P)H oxidase in vascular cells (28), and Ang II can alter VEGF expression and activity via activation of NADP(H) oxidase (54). We tested whether VEGF could be involved in the retinal leukostasis induced by Ang II. For this, Ang II was injected into the vitreal chamber together with SU5416, a small-molecular-weight inhibitor of the tyrosine kinase of VEGF receptors -1, -2, and -3 (38). Ang II-induced retinal leukostasis was markedly reduced by this treatment. Neutralizing antibodies against VEGF also suppressed the retinal leukostasis induced by ivt Ang II, indicating that
VEGF was involved in mediating Ang II-induced retinal leukostasis. Furthermore, thin frozen sections from retinas treated with ivt Ang II showed increased VEGF immunoreactivity, particularly in the ganglion cell layer. This suggests that the effects of Ang II involve activation of VEGF synthesis. Systemic infusion of Ang II has been reported to increase retinal VEGF (61), and now we show that increases in vitreal Ang II do likewise. These results suggest that the proinflammatory effects of Ang II in the retinal vasculature are associated with stimulation of VEGF synthesis in retinal cells and thus activation of VEGF receptor 2. In future studies, it would be interesting to precisely characterize the cellular components of the retina that respond to Ang II with superoxide-dependent increases in VEGF.

Induction of superoxide formation by Ang II was inferred through the effects of the antioxidants, in particular apocynin. To document changes in superoxide induced by Ang II in the retina, thin frozen sections of some retinas were loaded with the superoxide sensitive dye dihydroethidium (DHE). In the presence of superoxide, DHE is converted to oxyethidium that can be visualized by its high emitted red fluorescence. This reaction is sensitive and accurately reflects the presence of superoxide (23). Addition of Ang II causes a rapid and conspicuous increase in red fluorescence, which was accentuated in the outer and inner nuclear layers. In the retinal ganglion cell layer, only some isolated cells showed an increase in fluorescence that in some instances had a columnar appearance. A plausible interpretation of our findings is that Ang II causes activation of NAD(P)H oxidase and synthesis of superoxide. Increases in superoxide may stimulate VEGF activity, which in turn acts as a cytokine and exerts a pro-inflammatory effect on the retina. VEGF also activates NAD(P)H oxidase and thus superoxide formation (54). If this occurs in the retina, then Ang II and VEGF raise the levels of ROS, perhaps acting as a reinforcing loop. Previous studies have shown that the gp91phox-containing NAD(P)H oxidase is expressed in vascular endothelial cells (4;34). Furthermore, Ang II induces synthesis and release of a number of very active compounds (18, 23, 31, 23, 49). It may be that
the increased superoxide observed in the inner and outer nuclear layers after Ang II is due to some of these compounds.

Vascular inflammation is involved in the initiation and progression of diabetes-induced vascular complications. Current data suggest that Ang II modifies several steps in the inflammatory response (24). The data presented here suggest that Ang II, acting via the AT1 receptors, enhances ROS production in the retinal vascular wall through stimulation of NAD(P)H oxidase, leading to leukocyte infiltration, a pro-inflammatory response.

Increased oxidative stress induces a myriad of signaling cascades leading to expression of pro-inflammatory mediators and modification of the extracellular matrix as well as increased intracellular free Ca$^{2+}$ concentration which is a major determinant of vascular reactivity (6;48;50;54). Under pathological conditions, ROS contribute to vascular dysfunction and remodeling through oxidative damage. This occurs in diabetic retinopathy (30). There is growing evidence that oxygen free radicals are present in excess in diabetic retinas (19;26;35;36) and in vascular endothelial cells (49). Oxygen free radicals are toxic to tissue because of their high reactivity and ability to form covalent bonds non-enzymatically. It has been suggested that diabetic damage to retinas is caused by superoxide radicals (55).

Others and we have shown that inhibitors of the RAS suppress diabetes-induced retinal leukostasis (11;41). In addition Ang II and VEGF levels were significantly higher in patients with proliferative diabetic retinopathy than in non-diabetic patients or diabetic patients without retinopathy (25). This suggests that the renin-angiotensin system is involved in the pro-inflammatory effects of experimentally induced insulin-dependent diabetes in the retina. Since the pro-inflammatory effects of Ang II in the retina are suppressed by antioxidants and NAD(P)H oxidase inhibition, we assessed whether treatment with either a combination of tempol/NAC or apocynin decreased diabetes-induced retinal leukostasis. The results were very similar to those obtained for Ang II-induced retinal leukostasis. Both the general antioxidants and apocynin abolished diabetes-induced retinal leukostasis, consistent with data indicating that inhibition
of NAD(P)H oxidase activity blocks VEGF over-expression and neo-vascularization during ischemic retinopathy (4).

Taken together, these data suggest that development of retinal leukostasis in early diabetes requires an active RAS. Both Ang II- and diabetes-induced retinal leukostasis depend on the activity of NAD(P)H oxidase. Ang II may be crucial for NAD(P)H oxidase-derived superoxide and the resulting ROS to induce the changes that lead to retinal leukocyte infiltration in diabetes.

There are a number of questions that remain unanswered. For instance, we did not explore whether only superoxide or derived ROS such as H₂O₂ or peroxynitrate (47) are involved in the responses to Ang II and the development of diabetes. Superoxide formation reduces nitric oxide, and peroxynitrate can lead to tyrosine nitration and S-glutathiolation. We do not know the cellular (or subcellular) source of the NAD(P)H oxidase activity responsible for the effects shown here. At least two cell types within the retina have been suggested to express NAD(P)H oxidases: retinal pigment epithelium and retinal pericytes (6). However, the DHE data suggest that cells from the outer and inner retinal layers are the major source of the superoxide induced by Ang II, and indicating that they are targets for this peptide, either directly or indirectly through Ang II-released compounds that can activate NADPH oxidase (40-43, 62). In particular the strong reaction observed in the outer layer suggests that one or more of its cellular components responds to angiotensin II, or to a compound released by the peptide from some other cellular components, with increases in superoxide via NADPH oxidase activation. There are reports suggesting that angiotensin II is involved in diabetic neuropathies in the retina (8). Perhaps there is a functional relationship between angiotensin II, superoxide and retina neuronal function. Increased oxidative stress has been observed in the diabetic retina (45). Although we have documented that Ang II increases superoxide formation in the retina, at least in part by activating NAD(P)H oxidase activity, it is possible that in diabetes a deficiency in the retina antioxidant defense activity may be an important contributor to the enhanced oxidative stress.
The pro-inflammatory action of Ang II-NAD(P)H oxidase-superoxide may be one of the molecular mechanisms of diabetes-induced vascular retinal damage. A number of clinical studies have suggested that inhibiting the renin angiotensin system have a positive effect on progression of diabetic ocular complications (15;59). Thus drugs that interfere with the renin-angiotensin system may be useful to prevent or delay the progression of diabetic retinopathy.
Figure Legends

Fig 1. Intravitreal injection of Angiotensin II (Ang II, 20 µg) caused increased retinal leukostasis. Upper panel shows retinal leukostasis induced by Ang II alone or in the presence of the Ang II AT\textsubscript{1} receptor antagonist losartan or the Ang II AT\textsubscript{2} receptor antagonist PD123319. Ang II (20 µg) was injected ivt and static leukocytes in the peri-fundal area were detected 48 hr later using a scanning laser ophthalmoscope. Leukocytes were labeled with acridine orange. Those that remained arrested in the retinal microvasculature for at least 2 min were considered static (arrows). The retinal leukostasis induced by Ang II was inhibited by losartan but not by PD123319. These results are shown in the lower panel as M ± SEM. * = p < 0.01 vs control; # = p < 0.01 vs angiotensin II.

Fig 2. The retinal leukostasis induced by ivt Ang II was inhibited by the VEGFR2 kinase inhibitor SU5416 and by a blocking antibody against VEGF. * p < 0.001 vs control; # 0.01 vs angiotensin II

Fig 3. Immunoreactive VEGF was increased in retinas treated with ivt Ang II. Moderate changes in immunoreactivity were seen in cells of the retinal ganglia cell layer.

Fig 4. The effects of Ang II on retinal leukostasis were inhibited by tempol, a superoxide dismutase mimetic, N-acetyl- cysteine (NAC), a glutathione conserving antioxidant, and apocynin, an NAD(P)H oxidase inhibitor. Representative SLO images of leukocytes arrested in the retinal peri-fundal area (arrows) are shown in the upper panel. These data are shown in the lower panel as M ± SEM. * = p < 0.01 vs control; # p < 0.01 vs angiotensin II.

Fig 5. The effects of Ang II on retinal leukostasis were inhibited by tempol, NAC and apocynin, an NAD(P)H oxidase inhibitor. Representative images (x20)
of leukocytes arrested in the retinal veins are shown in the upper panel. A = control; B = angiotensin II; c = angiotensin II + tempol/NAC and d angiotensin II + apocynin. Vessels and leukocytes were stained by infusing FITC-concanavalin A and counting static leukocytes in retinal flat mounts (n = 4 in each group). The leukocytes appear as bright dots attached to the vascular intima which colocalize with CD45 immunoreactivity (right, x100). These data are shown in the lower panel. * = p < 0.01 vs control; # = p < 0.01, Ang II vs Ang II + treatments.

Fig 6. Fluorescent imaging of retinal sections incubated with dihydroethidium (DHE). Frozen sections (20 µm) sections were incubated with Ang II (1µM) alone, Ang II and apocynin (100 µM), or vehicle (0.1% EtOH). Thirty minutes later, the media was replaced by fresh solutions of these same compounds and DHE (2 µM). Incubation for a further 30min was followed by fluorescence microscopy and image captures. The figure shows (A) Control (B) apocynin alone (C) Ang II alone and (D) Ang II + Apocynin. Ang II induces marked increases in fluorescent intensity, indicating increased superoxide formation. The increases are visible in the Retinal ganglia cell layer (RGC), but they are quite marked on the inner nuclear layer (INL) and outer nuclear Layer (ONL). These increases were abolished by treatment with the NAD(P)H oxidase assembly inhibitor, apocynin, indicating that they are mediated by activation of NAD(P)H oxidase. The marked increase in DHE fluorescence induced by angiotensin II in the outer nuclear layer suggest that one or more of its cellular components responds either directly to angiotensin II or to a compound released by this peptide from some other cellular component with increases in NADPH oxidase-dependent superoxide.

Fig 7. The retinal leukostasis induced by diabetes was inhibited by tempol//NAC or apocynin. The upper panel shows that two weeks after induction of diabetes there was marked retinal leukostasis in the peri-fundal area, as determined using the SLO/acridine orange technique, which was markedly inhibited by the antioxidant treatments. Static leukocytes are indicated by arrows.
These data are shown in the lower panel ($n = 6$ in each group). * = $p < 0.01$ diabetes vs control; # $p < 0.01$ diabetes vs diabetes+ treatments

Fig 8. The increased number of static retinal leukocytes induced by two weeks of diabetes was inhibited by tempol/NAC or apocynin. The upper panel shows marked retinal leukostasis in the veins of the retina as determined by FITC-concanavalin A. The retinal leukostasis was markedly inhibited by the antioxidant treatments. These data are shown in the lower panel as $M \pm SD$ ($n = 3-5$ for each group). * = $p < 0.01$ vs control; # $p < 0.01$ diabetes vs treatments.
Acknowledgments. This work was supported by NEI 14385 and by the Alliance for Vision Research of MI to AGS. We are grateful to Gloria M Scicli for her expert assistance.
Reference List


41. Mori, F., T. Hikichi, T. Nagaoka, J. Takahashi, N. Kitaya, and A. Yoshida. Inhibitory effect of losartan, an AT1 angiotensin II receptor antagonist, on


Figure 2

The bar chart shows the static leukocytes per mm² for different treatments:
- Control
- Ang II
- Ang II + SU5416
- Ang II + VEGF antibody

Key:
- * indicates a significant difference compared to the control group.
- # indicates a significant difference compared to Ang II.
Figure 3
Figure 5

A

control

Ang II

Ang II +Tempo/NAC

Ang II + Apo

B

FITC Con A

CD 45

Merged

C

Retinal Leukostasis (Cells/Retina)

<table>
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<th></th>
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<th>Ang II + Tempol/NAC</th>
<th>Ang II + Apocynin</th>
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* p < 0.05 vs. control

# p < 0.05 vs. Ang II
Figure 6

Control

Ang II

Apocynin

Ang II + Apocynin
Figure 7

Normal Control  Diabetic  Diabetic + Tempol/NAC  Diabetic + Apocynin

Static Leukocytes Per mm²

Control  Diabetic  Diabetic + Tempol/NAC  Diabetic + Apocynin

*  #  *  #
Figure 8

Normal Control  Diabetic  Diabetic + Tempol/NAC  Diabetic + Apocynin

![Image of retinal images and bar graph showing comparison between normal control, diabetic, diabetic with Tempol/NAC, and diabetic with Apocynin. The bar graph indicates a significant increase in retinal leukostasis in diabetic rats compared to controls, with additional treatments showing reduced leukostasis.]

Retinal Leukostasis (Cells/Retina)

- Control
- Diabetic
- Diabetic + Tempol/NAC
- Diabetic + Apocynin

Significance:
- *: Significant difference compared to control
- #: Significant difference compared to diabetic alone

Bar graph values:
- Control: 10
- Diabetic: 70
- Diabetic + Tempol/NAC: 20
- Diabetic + Apocynin: 20