NAD\textsuperscript{+}-Induced Vasotoxicity in the Pericyte-Containing Microvasculature of the Rat Retina: Effect of Diabetes

Sophie D. Liao\textsuperscript{1} and Donald G. Puro\textsuperscript{1,2}

PURPOSE. It was recently proposed that activation of P2X\textsubscript{7} purinoceptors may play a role in causing cell death in the pericyte-containing microvasculature of the diabetic retina. This hypothesis is supported by the observation that diabetes enhances lethal pore formation in retinal microvessels exposed to synthetic P2X\textsubscript{7} agonists. The goal of this study was to determine whether purinergic vasotoxicity can be triggered by the endogenous molecule nicotinamide adenine dinucleotide (NAD\textsuperscript{+}), which is a substrate for ecto-ribosylation reactions known to activate P2X\textsubscript{7} receptor/channels in other cell types.

METHODS. Pericyte-containing retinal microvessels were isolated from normal and streptozotocin-injected rats. Trypan blue dye exclusion was used to assess cell viability. YO-PRO-1 uptake was used to identify cells with P2X\textsubscript{7}-induced pores, and etheno-adenosine antibodies were used to detect ecto-ADP-ribose moieties in retinal microvascular endothelial cells

RESULTS. In freshly isolated retinal microvessels, it was found that extracellular NAD\textsuperscript{+}, but not its catabolites, caused cell death (half-maximal effective concentration [EC\textsubscript{50} = 2 nM]) by a mechanism involving the activation of P2X\textsubscript{7} purinoceptors and the formation of transmembrane pores. A series of experiments provided evidence that NAD\textsuperscript{+}, which is not a direct purinergic agonist, serves as a substrate for ecto-ribosylation reactions that subsequently trigger P2X\textsubscript{7}-dependent cell death in the retinal microvasculature. Soon after the onset of diabetes, the sensitivity of retinal microvessels to the vasotoxic effect of extracellular NAD\textsuperscript{+} increased by approximately 100-fold.

CONCLUSIONS. Purinergic vasotoxicity triggered by extracellular NAD\textsuperscript{+} is a newly recognized mechanism that may contribute to the cell death observed in the pericyte-containing microvascular of the diabetic retina. (Invest Ophthalmol Vis Sci. 2006; 47:5032–5038) DOI:10.1167/iovs.06-0422

Cell death in pericyte-containing microvessels of the retina is a well-known complication of diabetes. However, the mechanism by which microvascular cell death occurs during the course of diabetic retinopathy remains incompletely understood. Recently, we suggested that P2X\textsubscript{7} purinoceptors may play a role.\textsuperscript{1} These ligand-gated receptor/channels are well known to be expressed by cells in the immune system,\textsuperscript{2} and we recently found that the pericyte-containing microvasculature of the rat retina also has functional P2X\textsubscript{7} purinoceptors.\textsuperscript{3}

When activated, these purinoceptors were found to cause the formation of large transmembrane pores,\textsuperscript{1,4} which may disrupt ionic gradients and allow the loss of vital intracellular molecules. Consistent with these potentially lethal events, we demonstrated previously that exposure of retinal microvessels to the synthetic P2X\textsubscript{7} agonist, benzoylbenzoyl-adenosine triphosphate (B\textsubscript{3}ATP), activates these purinoceptors, causes pores to open, and triggers apoptotic cell death.\textsuperscript{1,3,4} Thus, purinergic vasotoxicity may be a pathophysiologic mechanism in the retina.

We observed that the vulnerability of retinal microvessels to BzATP-induced vasotoxicity is markedly increased early in the course of streptozotocin-induced diabetes.\textsuperscript{1} This observation raised the possibility that purinergic vasotoxicity may mediate microvascular cell death in the diabetic retina. Unlike the synthetic P2X\textsubscript{7}-analog BzATP, however, we found that exposure of retinal microvessels to the endogenous purinergic ligand ATP did not result in pore formation, despite its activation of P2X\textsubscript{7} purinoceptors.\textsuperscript{4} Subsequently, we determined that the basis for this unexpected observation was that ATP is an agonist not only for P2X\textsubscript{7} purinoceptors but also for P2Y\textsubscript{4} purinoceptors, whose activation triggers a cascade of events that inhibit the formation of P2X\textsubscript{7} pores in normal and diabetic retinal microvessels.\textsuperscript{4} Based on these findings with ATP, we concluded that if purinergic vasotoxicity plays a role in diabetic retinopathy, P2X\textsubscript{7} purinoceptors must be activated by another endogenous molecule that does not simultaneously prevent pore formation.

In this study, we considered the possibility that purinergic vasotoxicity in retinal microvessels can be induced by extracellular nicotinamide adenine dinucleotide (NAD\textsuperscript{+}). This nucleotide was of interest because of its recently elucidated role in the P2X\textsubscript{7}-dependent death of immune T cells. As reported by others,\textsuperscript{5,7} P2X\textsubscript{7} purinoceptors on T cells are activated by a mechanism involving ribosylation reactions catalyzed by an ecto-ADP-ribosyltransferase (ART) that uses extracellular NAD\textsuperscript{+} as a substrate. Based on these studies, we investigated whether a similar mechanism could occur in retinas in which microvascular pericytes and endothelial cells also express P2X\textsubscript{7} purinoceptors\textsuperscript{5} and in which NAD\textsuperscript{+} may be an important extracellular signaling molecule.\textsuperscript{8}

We now report that extracellular NAD\textsuperscript{+} induces vasotoxicity in the pericyte-containing retinal microvasculature. This NAD\textsuperscript{+}-induced cell death was associated with the formation of transmembrane pores and was blocked by P2X\textsubscript{7} antagonists. Consistent with the presence of an ecto-ART that links NAD\textsuperscript{+} exposure with P2X\textsubscript{7}-mediated cell death, we detected ART activity on retinal microvessels. Indicative of the importance of ART, NAD\textsuperscript{+}-induced vasotoxicity was blocked by etheno-NAD\textsuperscript{+}, which is a competitive ART substrate whose donated etheno-ADP-ribose moieties do not trigger P2X\textsubscript{7}-dependent cell death.\textsuperscript{3} We found that soon after the onset of streptozotocin-induced diabetes, the sensitivity of the retinal microvasculature to NAD\textsuperscript{+}-induced cell death increased approximately 100-fold.
METHODS

Procedures regarding animal use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Michigan Committee on the Use and Care of Animals. Long-Evans rats (Charles River, Cambridge, MA) were maintained on a 12-hour alternating light/dark cycle and received food and water ad libitum.

Microvessel Isolation

Six- to 14-week-old rats were killed with increasing concentration of carbon dioxide. After rapid removal from the eye, retinas were incubated for 24 minutes at 30°C in 2.5 mL Earle balanced salt solution supplemented with 0.5 mM EDTA, 20 mM glucose, 6 to 8 U papain (Worthington Biochemicals, Freehold, NJ), and 2 mM cysteine. To maintain pH and oxygenation, 95% oxygen/5% carbon dioxide was bubbled into this solution for approximately 4 minutes. For each new lot of papain, the amount of enzyme and the duration of incubation were empirically adjusted to optimize the yield of isolated microvessels. After incubation in the papain-containing solution, retinas were 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, with osmolarity adjusted to 310 mOsm L⁻¹. Retinas were then cut into quadrants, and each piece was gently sandwiched between two glass coverslips (15-mm diameter; Warner Instrument Corp., Hamden, CT) that had been soaked in 50% ethanol. Retinas were then cut into quadrants, and each piece was gently sandwiched between two glass coverslips (15-mm diameter; Warner Instrument Corp., Hamden, CT) that had been soaked in 50% ethanol for 30 minutes and then washed in distilled water. Retinal vessels adhered to the coverslip in contact with the vitreal side of the retina; photomicrographs of freshly isolated retinal microvessels appear in several publications.⁹⁻¹²

Pericyte-containing microvessels were identified by the “bump on a log” appearance of the abluminal pericytes.⁶⁻¹²¹³ The pericyte-containing retinal microvascular includes the microvessels distal to smooth muscle-encircled arterioles and proximal to venules; some authors have subclassified this microvascular into metarterioles, precapillaries, micropapillaries, and capillaries. Experimental findings reported here include observations from all portions of the pericyte-containing microvasculature of the retina.

Model of Diabetes

After an overnight fast, 5- to 6-week-old Long-Evans rats received intraperitoneal injection of streptozotocin (STZ) diluted in 0.8 mL citrate buffer. Early in this study, rats received 75 mg kg⁻¹ STZ. However, because hyperglycemia developed in only approximately 50% of the rats, the dose was increased to 150 mg kg⁻¹, which caused persistently high blood glucose levels in all injected animals. For the initial 24 hours after streptozotocin injection, glucose-supplemented water was provided to minimize possible hypoglycemic episodes that might have occurred secondary to the lysis of pancreatic β cells. The diabetic rats used in this study were hyperglycemic for 5 weeks or less (20.1 ± 8.2 days); insulin was not administered. Immediately before the retinal microvessels were harvested, the blood glucose level of the diabetic rats was 358 ± 18 mg/dL (n = 16).

Cell Viability Assay

Microvascular cells that failed to exclude trypan blue dye were classified as dead. To perform this assay, coverslips containing retinal microvessels were exposed for 15 minutes to 0.04% trypan blue in solution A. Microvessels were then promptly examined at ×100 magnification with an inverted microscope equipped with bright-field optics, and the percentage of cells stained with trypan blue was determined. Immediately after the isolation of microvessels from the retina, the viability of at least 200 cells per coverslip was determined. Assessed microvessels were at least 300 μm long. After this initial count, the location on each coverslip of the assayed microvessels was documented to facilitate the subsequent reassessment of cell viability in the same microvascular complexes.

Various experimental protocols were used in the cell death studies. Experiments were conducted at 36° to 37°C. In experiments using solution A supplemented with UTP, freshly isolated microvessels were exposed to this nucleotide for 30 minutes before an additional 24-hour exposure to solution A plus UTP without or with nicotinamide adenine dinucleotide (NAD⁺). In experiments using brilliant blue or etheno-NAD⁺, these chemicals were in solution A for 1 hour before NAD⁺ or benzoylbenzoyl-ATP (BzATP) was added. In experiments using oxidized-ATP, microvessels were incubated in solution A supplemented with this P2X- antagonist for 2 hours before the addition of NAD⁺. Unless noted otherwise, we determined the percentage of trypan blue–positive cells after 24-hour exposure of the retinal microvessels to NAD⁺.

NAD⁺-Induced Vasotoxicity

As in our previous studies,¹⁻³⁴ cells with P2X₇ pores were detected using YO-PRO-1 (Molecular Probes, Eugene, OR), which is a 629-Da propidium diiodide dye that passes through these pores and produces detectable fluorescence when it binds to nuclear DNA. Unless stated otherwise, a microvessel-containing coverslip was initially exposed for 30 minutes to solution A supplemented with 5 μM YO-PRO-1 and then positioned in a chamber located on the stage of a microscope (Eclipse E600 FN; Nikon, Tokyo, Japan) equipped with ×10 and ×40 objectives for fluorescence and bright-field microscopy. Fluorescence was detected with excitation and emission wavelengths of 488 and 510 nm, respectively; the light source was a xenon arc system (Lambda-LS; Sutter Instruments, Novato, CA). Digitized images were obtained and stored using a system that included an intensified charge-coupled device camera with a 12-bit dynamic range (CoolSnap HQ; Photometrics, Tucson, AZ) and software (MetaMorph; Molecular Devices, Downingtown, PA). After 30-minute incubation in YO-PRO-1, 14.5% (n = 674) of the nondiabetic and 14.6% (n = 628) of the diabetic microvascular cells were positive for this dye; occasional microvessels with more than 50% of YO-PRO–positive cells were not studied further. After the initial 30 minutes of exposure to solution A supplemented with 5 μM YO-PRO-1, microvessels were incubated for 60 minutes in the same YO-PRO–containing solution without or with added NAD⁺. Subsequently, we again obtained images of the same microvascular complexes that were photographed previously. From the digitized images, the total percentage of YO-PRO–positive cells in the selected microvessels was again determined (see Figs. 2D, 4C). In the absence of added NAD⁺, 16.7% of the microvascular cells were YO-PRO positive after 90 minutes in solution A supplemented with 5 μM YO-PRO-1; this was not significantly different (P = 0.3) from the 14.6% detected after the initial 30-minute incubation. In diabetic microvessels, the YO-PRO positivity in the absence of NAD⁺ was 14.6% (n = 628) after 30 minutes in the presence of this fluorescent probe and 16.1% (n = 628) 60 minutes later; with the nondiabetic microvessels, the uptake of YO-PRO-1 did not significantly change between 30 and 90 minutes of exposure to 5 μM YO-PRO-1 in solution A.

Immunodetection of Cell Surface ADP-ribosylation

Using an antibody specific for ethenoadenosine to label ribosylated moieties,¹⁸ we detected ADP-ribosyltransferase activity after exposing freshly isolated retinal microvessels to etheno-NAD⁺. Unless noted otherwise, steps in this assay were performed at room temperature. After exposure to 10 nM etheno-NAD⁺ in solution A for 5 hours at 36°C to 37°C, microvessels were fixed by air drying for 1 hour, incubated in 0.3% hydrogen peroxide for 30 minutes, and exposed for 16 hours at 4°C to a mouse anti-ethenoadenosine antibody (clone 1G4; Novus...
Biologicals, Littleton, CO) that was diluted 1:100 in PBS. In controls, no primary antibody was used. Subsequently, coverslips were exposed for 1 hour to PBS supplemented with biotin-conjugated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) and 1.5% horse serum. After they were washed in PBS, the microvessels were kept in a horseradish peroxidase–streptavidin solution (RTU; Vector Laboratories) for 50 hours at 4°C and exposed to the avidin-biotin-peroxidase complex (ABC method; Vector Laboratories) at 1:100 for 30 minutes. After development with diaminobenzidine, microvessels were examined with bright-field optics.

**Chemicals**

Unless noted otherwise, chemicals were obtained from Sigma (St. Louis, MO).

**Statistical Analysis**

Standard deviations are presented in the text and shown in the figures. Unless noted otherwise, probability was evaluated by analysis of variance (ANOVA). For comparisons between two groups, the Student’s t-test or the Fisher exact test were used, as appropriate.

**RESULTS**

**NAD⁺-Induced Cell Death in Retinal Microvessels**

To test the hypothesis that NAD⁺ induces vasotoxicity in the retinal microvasculature, we exposed microvessels freshly isolated from rat retinas to various concentrations of this chemical. After 24 hours, cell viability in the retinal microvasculature was assayed using the trypan blue dye exclusion test. As shown in Figure 1A, exposure to NAD⁺ increased microvascular cell death in a dose-dependent manner; the half-maximally effective concentration of NAD⁺ was approximately 2 nM. An assessment of the time course for NAD⁺-induced vasotoxicity revealed that cell death was detected after 16 hours of exposure to NAD⁺ (Fig. 1B). Of interest, sustained exposure to NAD⁺ was not required to trigger microvascular cell death. For example, we found that 24 hours after a 10-minute exposure to 10 nM NAD⁺, 33.2 ± 13.1% (n = 10) of the microvascular cells were trypan blue positive. This was significantly (P < 0.001) greater than the 10.7% ± 5.7% (n = 67) rate of cell death in retinal microvessels maintained for 24 hours in an NAD⁺-free solution.

By what mechanism does exposure to extracellular NAD⁺ trigger cell death in pericyte-containing retinal microvessels? We focused on the possible role of P2X₇ purinoceptors because studies by others² have shown that P2X₇ activation is a key step in NAD⁺-induced death of immune T cells. Furthermore, an association between NAD⁺ and P2X₇ purinoceptors was of interest in our study of retinal microvessels because we recently demonstrated that these purinoceptors are functional in the retinal microvasculature³ and that their activation can cause microvascular cell death.¹⁴ Consistent with P2X₇ purinoceptors playing an essential role in mediating NAD⁺-induced vasotoxicity, the microvascular cell death induced by NAD⁺ was markedly decreased by brilliant blue (P < 0.001) and oxidized ATP (P = 0.001), which are P2X₇ antagonists (Fig. 1C).

**NAD⁺-Induced Pore Formation**

Previously, we reported that the selective activation of P2X₇ purinoceptors is associated with the formation in retinal microvascular cells of transmembrane pores,¹³ which may disrupt ionic gradients and cause the loss of vital intracellular molecules. To determine whether exposure to NAD⁺ results in pore formation, we exposed microvessels to NAD⁺ and monitored the uptake by microvascular cells of Yo-PRO-1, which is a 629-Da fluorescent dye that passes through P2X₇ pores.² In agreement with P2X₇ pores forming during NAD⁺ exposure, the percentage of microvascular cells containing Yo-PRO-1 was markedly (P < 0.001; Fisher exact test) increased in pericyte-containing microvessels incubated in the presence of 10 nM NAD⁺ (Fig. 2). Consistent with the role of P2X₇ purinoceptors in mediating the formation of pores, the NAD⁺-induced increase in Yo-PRO-1 uptake was prevented (P < 0.001; Fisher exact test) by 100 nM brilliant blue, which is a P2X₇ antagonist. Further support for the importance of pore formation was our observation that NAD⁺-induced vasotoxicity was negligible (P < 0.001) in retinal microvessels pre-exposed to extracellular uridine triphosphate (UTP; Fig. 1C), whose activation of P2Y₄ purinoceptors inhibits the formation of P2X₇ pores in the retinal microvasculature.⁴ From these experimental results, we concluded that extracellular NAD⁺

![Figure 1. NAD⁺-induced microvascular cell death. (A) Cell death detected by trypan blue dye exclusion in retinal microvessels exposed for 24 hours to various concentrations of NAD⁺. For NAD⁺ concentrations ≥2 nM, cell death was significantly (P < 0.001, Student’s t-test) increased. In the group of microvessels exposed to solution A without NAD⁺, 67 vessel-containing coverslips were assessed; for each of the other groups, 6.3 ± 1.5 vessel-containing coverslips were assessed. Coverslips were used. (B) Time course of cell death during exposure of retinal microvessels to solution A with (●) or without (▲) 10 nM NAD⁺. Cell viability at time 0 was determined from 82 vessel-containing coverslips. The 24-hour value for the no NAD⁺ group was determined from an assessment of 67 vessel-containing coverslips. For the other time points, 7 ± 2.4 microvessel-containing coverslips were assessed. (C) Microvascular cell death under a variety of experimental conditions: (1) solution A only (no additives), n = 67; (2) 10 nM NAD⁺, n = 8; (3) 10 nM NAD⁺ plus 100 nM brilliant blue (BB), which is a P2X₇ antagonist, n = 5; (4) 10 nM NAD⁺ plus 300 μM oxidized ATP (oxATP), which is another P2X₇ antagonist, n = 5; (5) 10 nM NAD⁺ plus 100 μM UTP, whose activation of P2Y₁ purinoceptors prevents pores from forming during P2X₇ activation², n = 4; (6) 1 mM ADP+ribose, which is an NAD⁺ catabolite, n = 4; (7) 1 mM nicotinamide, which is another NAD⁺ catabolite, n = 5; (8) 100 μM etheno-NAD⁺ (enNAD⁺), which is an analog of NAD⁺, n = 6; (9) 10 nM NAD⁺ plus 100 μM etheno-NAD⁺, n = 6; (10) the P2X₇ antagonist benzoylbenzoylATP (BzATP, 100 μM) plus 100 μM etheno-NAD⁺, n = 6. The null hypothesis that the means of the 10 groups were identical was rejected (P < 0.001) by ANOVA. Using the protected least significant difference approach to adjust for multiple comparisons, post hoc testing of the difference between pairs of means showed that the mean of the NAD⁺ group differed significantly from that of all other groups (P < 0.001) in each paired comparison, except for the BzATP/enNAD⁺ group (P = 0.4); similarly, the no additives group differed significantly (P < 0.001) from the NAD⁺ and BzATP/enNAD groups but was not significantly different (P > 0.3) from the other groups.
FIGURE 2. Effect of NAD\textsuperscript{+} on the uptake of YO-PRO-1. (A) Differential interference contrast photomicrograph of a freshly isolated microvascular complex. (B) Photomicrograph of the same microvascular complex as in (A) showing fluorescence detected 30 minutes after incubation in solution A supplemented with 5 μM YO-PRO-1. (C) Photomicrograph of the same microvessels after subsequent 60-minute exposure to the YO-PRO-1 solution plus 10 nM NAD\textsuperscript{+}. (D) Percentage of cells with YO-PRO-stained nuclei. Microvessels were first incubated for 30 minutes in solution A containing 5 μM YO-PRO-1 with or without 100 nM brilliant blue, subsequently exposed for 60 minutes to the same solution that was without or with added 10 nM NAD\textsuperscript{+}, and then assessed for YO-PRO-positivity. For the no NAD\textsuperscript{+}, NAD\textsuperscript{+}, and NAD\textsuperscript{+}/BB groups, 672, 709, and 182 microvascular cells, respectively, were assessed for YO-PRO uptake. NAD\textsuperscript{+} significantly (P < 0.001; Fisher exact test) increased YO-PRO uptake, and brilliant blue significantly (P < 0.001; Fisher exact test) decreased NAD\textsuperscript{+}-induced uptake of YO-PRO-1.

CAUSES CELL DEATH IN PERICYTE-CONTAINING RETINAL MICROVESSELS BY A MECHANISM INVOLVING THE ACTIVATION OF P2X\textsubscript{7} PURINOCEPTORS AND THE FORMATION OF LETHAL PORES.

ADP-ribosylation and Purinergic Vasotoxicity

How does exposure of retinal microvessels to NAD\textsuperscript{+}, which is not a ligand for P2X\textsubscript{7} purinoceptors, result in the activation of these receptors? One possibility we initially considered was that catabolites of NAD\textsuperscript{+}, rather than NAD\textsuperscript{+} itself, are vaso-toxic. However, as shown in Figure 1C, exposure of freshly isolated retinal microvessels to ADP-ribose (1 mM) or nicotinamide (1 mM) did not significantly (P > 0.3) increase cell death. Thus, we concluded that NAD\textsuperscript{+} initiates a series of events resulting in P2X\textsubscript{7} activation, pore formation, and cell death.

To help elucidate the mechanism by which NAD\textsuperscript{+} induces cell death in the retinal microvasculature, we performed a series of experiments that were based on previous studies of the NAD\textsuperscript{+}-induced death of immune T cells. Koch-Nolte et al.\textsuperscript{5} recently demonstrated that the killing of T cells by NAD\textsuperscript{+} is dependent on ecto-ART, which uses NAD\textsuperscript{+} as a substrate for ribosylation reactions that result in the activation of P2X\textsubscript{7} purinoceptors. To begin to assess whether a similar mechanism could occur in the microvasculature of the retina, we used an immunoassay to determine whether these vessels have ecto-ART activity. Consistent with this possibility, we detected ART activity in pericyte-containing retinal microvessels that had been initially exposed to the NAD\textsuperscript{+} analog, etheno-NAD\textsuperscript{+} and subsequently were probed with an antibody specific for ribosylated moieties containing ethenoadenosine (Fig. 3).\textsuperscript{15} Although specific ART inhibitors have not yet been discovered,\textsuperscript{16} we attempted to further assess the putative role of ART in NAD\textsuperscript{+}-induced vasotoxicity by exposing microvessels to a variety of nonspecific inhibitors of this type of enzyme. However, the nonspecific ART inhibitors we tested—novobiocin (500 μM), 1.8-naphthalamide (100 μM), vitamin K\textsubscript{1} (100 μM), vitamin K\textsubscript{2} (100 μM), and vitamin K\textsubscript{3} (1 mM)—were themselves highly toxic to retinal microvascular cells.

In contrast to these unsuccessful experiments with nonspecific inhibitors, the NAD\textsuperscript{+} analog etheno-NAD\textsuperscript{+}, which is an ART substrate, proved useful for assessing the role of ribosylation in linking NAD\textsuperscript{+} exposure with P2X\textsubscript{7}-dependent vasotoxicity. Similar to findings with T cells,\textsuperscript{5} we observed that this NAD\textsuperscript{+} analog neither induced microvascular cell death (Fig. 1C) nor blocked the lethal effect of the P2X\textsubscript{7} ligand BzATP (Fig. 1C). Presumably, structural features of the P2X\textsubscript{7} receptor binding site prevent etheno-ADP-riboyl moieties from activating this purinoceptor or from blocking the interaction of BzATP with these receptor/channels.\textsuperscript{5} Hence, nonlethal etheno-NAD\textsuperscript{+} can be used to compete with NAD\textsuperscript{+} as a substrate for ART.\textsuperscript{5} In agreement with ART-mediated ribosylation playing a role in the vasotoxic effect of NAD\textsuperscript{+}, we found that preincubation of retinal microvessels with etheno-NAD\textsuperscript{+} prevented (P < 0.001) cell death induced by NAD\textsuperscript{+} (Fig. 1C). Thus, our experiments with etheno-NAD\textsuperscript{+} support the hypothesis that the vasotoxicity of extracellular NAD\textsuperscript{+} involves ecto-ribosylation.

NAD\textsuperscript{+}-Induced Vasotoxicity in Diabetic Microvessels

Because an important feature of diabetic retinopathy is the death of cells within the pericyte-containing microvasculature, we also assessed the effect of NAD\textsuperscript{+} on retinal microvessels freshly isolated from rats made diabetic by streptozotocin-injection. Figure 4A shows that there was a marked shift to the left in the dose–response relationship for NAD\textsuperscript{+}-induced cell death in diabetic, compared with nondiabetic, retinal microvessels. As shown in Figure 4B, the increase in vulnerability of diabetic microvessels to the vasotoxic effect of NAD\textsuperscript{+} was detected early in the course of insulin-deficient diabetes. In agreement with this vasotoxicity in diabetic microvessels being mediated by P2X\textsubscript{7} purinoceptors, the P2X\textsubscript{7} antagonist–oxidized ATP (300 μM) prevented NAD\textsuperscript{+}-induced cell death. More specifically, the rate of cell death was only 5.7% ± 2.0% (n = 3), which was not significantly different (P = 0.06) from the
control rate of 9.9% ± 7.3% (n = 21), when microvessels harvested 17 days after streptozotocin injection were incubated in the presence of the P2X7 antagonist plus 10 nM NAD+, which is an NAD+ concentration that is approximately 1000-fold greater than its half-maximal effective concentration (EC50) for killing diabetic microvascular cells (Fig. 4A).

Consistent with diabetes increasing the sensitivity of the retinal microvasculature to NAD+ -induced cell death, we observed that 1 nM NAD+ was markedly more effective in increasing the formation of pores in diabetic, compared with nondiabetic, microvessels. As shown in Figure 4C, 47.8% of the cells (n = 890) in diabetic microvessels were YO-PRO positive after exposure to 1 nM NAD+. This is significantly (P < 0.001; Fisher exact test) greater than the 20.7% detected in nondiabetic microvessels. Of note, in this study of diabetic microvessels, we used 1 nM NAD+. In contrast, Figure 2C shows results of nondiabetic microvessels exposed to 10 nM NAD+. For the nondiabetic/NAD+/BB groups, 984, 890, and 1121 microvascular cells, respectively, were assessed for YO-PRO uptake. Diabetic microvessels were obtained 10 ± 1 days after rats were injected with streptozotocin.

**Comparisons of Nondiabetic and Diabetic Microvessels**

We considered the possibility that the diabetes-induced increase in lethality of NAD+ in the pericyte-containing retinal microvasculature was caused by a nonspecific propensity for isolated diabetic microvessels to die. However, similar to our previous assessments, we found in this study that immediately after the isolation of retinal microvessels, rates of cell death were 4.8% ± 2.4% (n = 597) and 4.7% ± 2.1% (n = 112) for nondiabetic and diabetic microvessels, respectively; these values are not significantly different (P = 0.7; Student t test). In addition, after incubation for 24 hours in solution A, microvessels from normal and diabetic retinas underwent similar (P = 0.6; Student t test) rates of cell death—10.7% ± 5.7% (n = 67 coverslips) and 9.9% ± 7.3% (n = 21), respectively. Furthermore, after incubation for 90 minutes in solution A supplemented with 5 μM YO-PRO-1, the percentage of cells that became YO-PRO positive was not significantly different (P = 0.8; Fisher exact test) in nondiabetic (4.7%; n = 672) and diabetic (16.1%; n = 628) microvessels. Taken together, these observations indicate that streptozotocin-induced diabetes did not decrease the viability or increase the YO-PRO-1 permeability of cells in isolated retinal microvessels.

Our experimental results support the idea that soon after the onset of insulin-deficient diabetes, pericyte-containing microvessels of the retina become markedly more vulnerable to NAD+ -induced vasotoxicity, which our experiments indicate is mediated by a mechanism involving ribosylation, P2X7 activation, and pore formation.

**DISCUSSION**

This study shows that extracellular NAD+ triggers a cascade of events that induce cell death in the pericyte-containing microvasculature of the rat retina. Indicative of the importance of NAD+, the metabolic breakdown products of NAD+, ADP-ribose, and nicotinamide, were not vasotoxic. Our findings support the hypothesis that the vasotoxicity of NAD+ is mediated by P2X7 purinoceptors whose activation results in the formation of transmembrane pores. Consistent with the lethality of these pores, NAD+ -induced cell death was minimal in the presence of UTP, which, by activating P2Y1 purinoceptors, initiates events that block the P2X7 channel-to-pore transition in retinal microvascular cells. To account for P2X7-dependent death being induced by NAD+, which is not a direct P2X7 agonist, we investigated the possibility that this nucleotide serves as a substrate for ribosylation reactions that activate P2X7 purinoceptors, thereby causing lethal pores to form. Supporting the feasibility of this putative mechanism, we detected ART activity on the surfaces of pericyte-containing retinal microvessels. Further evidence for ART playing a critical role is provided by our finding that the vasotoxicity of NAD+ was blocked by etheno-NAD+, which is a competing ART substrate that does not induce cell death. Of pathophysiologic interest, the vasotoxic effects of NAD+ were markedly increased in diabetic microvessels, which, as we previously reported, show an increase in the transition from activated P2X7 channel to opened pore. An unexpected finding in our study was the remarkable sensitivity of the retinal microvasculature to extracellular NAD+. In microvessels isolated from the normal retina, the...
EC_50 for NAD\(^+\) was 2 nM. In contrast, an approximately 1000-fold greater NAD\(^+\) concentration is required to kill T cells,\(^5\) which are the only other cells assessed for P2X-dependent NAD\(^+\)-induced death. At present, the reasons for the marked difference in the sensitivity of microvascular and immune cells to NAD\(^+\) are uncertain. One possibility is that the ecto-ART of T cells, compared with that of vascular cells, has a lower affinity for NAD\(^+\); however, because the Michaelis-Menten constants for the ecto-ARTs of T cells and vascular cells have not been determined, this possibility remains to be rigorously assessed. On the other hand, in contrast to the micromolar concentrations of NAD\(^+\) required by the ART of T cells,\(^5,17,18\) cultured vascular endothelial cells are reported to robustly ribosylate a soluble protein at room temperature in the presence of only 125 nM NAD\(^+\).\(^13\) Furthermore, based on biochemical studies of various ARTs,\(^20\) it seems likely that an even lower NAD\(^+\) concentration would be effectively used by the endothelial ART when the ribosylation target is membrane bound rather than in solution and when the reaction occurs at 37\(^\circ\) rather than at room temperature. Another possible explanation for the higher sensitivity of microvessels to NAD\(^+\) may be that catalysis of this nucleotide by microvessels is minimal. Suggestive of this, Dianzani et al.\(^21\) reported that vascular endothelial cells do not express the potent ecto-NAD\(^+\)-glycohydrolase CD38. In contrast, T cells do express CD38, which has been demonstrated to significantly limit the ability of these immune cells to subject ecto-proteins to ADP-ribosylation.\(^18\) A goal for future studies is to elucidate the basis for the exquisite sensitivity of pericyte-containing retinal microvessels to extracellular NAD\(^+\).

What are the sources for extracellular NAD\(^+\)? Although not excluding the possibility that NAD\(^+\) synthesis occurs on the cell surface, essentially all attention has focused on the release of intracellular NAD\(^+\), which is present at millimolar concentrations. One demonstrated mechanism by which NAD\(^+\) exits cells is through connexin 43 hemichannels.\(^22\) Although no study has been published of NAD\(^+\) efflux from microvascular cells, this mechanism may be of relevance because retinal pericytes express this connexin.\(^22\) Another mechanism for the release of NAD\(^+\) may be mechanical forces causing a transient loss in the integrity of the plasma membrane. This process—cell wounding—has been described in skeletal and cardiac muscle cells\(^23\) and perhaps may occur in vascular endothelial cells because they endure shear forces or in pericytes as they contract. Another potential source of extracellular NAD\(^+\) is plasma, which contains approximately 200 nM of this nucleotide. In addition, under pathologic conditions, it is likely that the lysis of cells would release significant amounts of NAD\(^+\) into the extracellular milieu.

Although the principal finding of this study is that extracellular NAD\(^+\) is vasotoxic, we postulate that in the normal retina, vasotoxicity must be prevented to avoid sight-threatening consequences. One likely protective mechanism is the catabolism of extracellular NAD\(^+\). Consistent with this scenario, the ecto-NAD\(^+\)-glycohydrolase CD38 is located on the surface of the Müller (glial) cells,\(^8\) whose processes ensheath the retinal vasculature. In addition, tight junctions between endothelial cells of the retinal vasculature establish a barrier that prevents access to the retinal extracellular space of plasma-containing NAD\(^+\). Another likely mechanism to protect against NAD\(^+\)-induced vasotoxicity is inhibition of pore formation. Recently, we reported that the activation of P2Y_4 purinoceptors by extracellular ATP or UTP prevents P2X_7 pores from opening.\(^4\) Although a retinal source for extracellular UTP remains to be identified, this protective mechanism may be triggered as a consequence of Müller cells releasing ATP,\(^25\) which is thought to serve as a glial-to-vascular signal. In this way, extracellular ATP may not only have a vasoactive function in the retina, it may also play a vasoprotective role.

Because the formation of potentially lethal P2X_7 pores is almost certainly tightly regulated, we suspect that extracellular NAD\(^+\) has a physiologic function in the retinal microvasculature. For example, the NAD\(^+\)-induced activation of P2X_7 receptor/channels provides pathways for the influx of calcium, which causes pericytes to contract and, thereby, lumens to constrict.\(^3,26\) In addition, NAD\(^+\) is a precursor of catabolic products that may also regulate calcium levels in retinal vascular cells. On the other hand, we predict that NAD\(^+\)-induced vasotoxicity occurs in the retinal microvasculature under certain pathologic conditions, such as when plasma-derived NAD\(^+\) enters the retina at sites of a diabetes-induced breakdown of the blood-retinal barrier, when hyperglycemia up-regulates ecto-ADP-ribosylation,\(^27\) when autoantibodies block CD38 activity in patients with diabetes,\(^28\) or when diabetes enhances the formation of P2X_7-induced pores.\(^1\)

Our conclusions concerning the mechanism by which NAD\(^+\) induces P2X-dependent vasotoxicity are based on experiments using pericyte-containing microvessels freshly isolated from the rat retina. A benefit of studying microvessels in isolation is that potentially confounding effects of NAD\(^+\) or its catabolites on nonvascular retinal cells can be eliminated. In addition, the concentration of NAD\(^+\) and the duration of its exposure to microvessels can be well controlled. On the other hand, it remains to be demonstrated that extracellular NAD\(^+\) triggers cell death in retinal microvessels in vivo. In addition, the possibility of species differences in microvascular ARTs or P2X_7 purinoceptors warrants caution in extrapolating our findings to the pathophysiology of diabetes in humans. Additionally, because of our inability to reliably distinguish between swollen, trypan blue–positive endothelial cells and stained pericytes, we cannot assess whether both types of microvascular cells are equally vulnerable to purinergic vasotoxicity even though our immunocytochemistry\(^3\) and electrophysiology (Yamanishi S, Puro DG, unpublished data, October 2003) studies suggest that both pericytes and endothelial cells express P2X_7, purinoceptors.

In summary, despite caveats, freshly isolated microvessels are useful in the testing of hypotheses concerning pathophysiologic mechanisms mediating microvascular complications of diabetes.\(^29\) Based on our experimental findings, we propose that diabetes markedly increases the sensitivity of the pericyte-containing retinal microvasculature to NAD\(^+\)-induced vasotoxicity. Thus, purinergic vasotoxicity triggered by extracellular NAD\(^+\) may play a role in the microvascular cell death associated with diabetic retinopathy. If so, then selectively interfering with ribosylation, P2X_7 activation, or pore formation in the pericyte-containing retinal microvasculature may be of benefit in ameliorating sight-threatening complications of diabetes.

Acknowledgments

The authors thank Takatoshi Kobayashi and Kenji Matsushita for providing technical help. Bret Hughes for generously allowing use of equipment, and David Musch for help with statistical analysis.

References


