Role of NADPH Oxidase in Retinal Microvascular Permeability Increase by RAGE Activation

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PURPOSE. The accumulation of advanced glycation end products (AGEs) within the retina in diabetes is associated with a chronic increase in retinal microvascular permeability. Isolated perfused retinas were used to examine the acute effects of AGEs on retinal microvascular permeability.

METHODS. Retinas were dissected from eyes obtained from male Wistar rats, pinned out flat, and perfused with the low-molecular-weight fluorescent dye sulforhodamine B. Microvascular permeability was determined from the rate of decrease in fluorescence gradient across a vessel under conditions of zero flow. The production of reactive oxygen species (ROS) in JG2.1 retinal endothelial cells was also assessed with a fluorescent probe working solution.

RESULTS. A 30-second application of AGE-modified bovine serum albumin (AGE-BSA) to the abluminal surface of the retinal vasculature produced a rapid dose-dependent increase in retinal capillary permeability that was inhibited by pretreatment with anti-RAGE IgG. The permeability response also required ROS generated by NADPH oxidase because pretreatment with apocynin and the free radical scavengers superoxide dismutase and catalase significantly reduced the response. Pretreatment with calphostin C, SKF-96365, and U-73122 also significantly reduced the permeability response. In addition, the permeability response to bradykinin increased permeability through ROS and was potentiated after pretreatment with AGE-BSA. This potentiation was blocked by apocynin.

CONCLUSIONS. Acute activation of NADPH oxidase by phospholipase C-mediated activation of Ca2+-dependent PKC occurs downstream of RAGE activation to acutely increase retinal capillary permeability in the isolated perfused rat retina. (Invest Ophthalmol Vis Sci. 2009;50:1319–1328) DOI:10.1167/iovs.08-2730

Vision loss in patients with type 2 diabetes occurs predominantly as a consequence of macular edema, which results after breakdown of the blood-retinal barrier (BRB).1 Fluorescein leakage caused by increased vascular permeability is one of the earliest detectable findings in diabetic retinopathy, as determined by vitreous fluorophotometry2 and fluorescein angiography,3 and precedes the appearance of microaneurysms, capillary loss, and vessel abnormalities. Increased permeability of the BRB, which is localized to the tight junctions between adjacent endothelial cells,4 may therefore be considered a crucial factor in the initiation and progression of diabetic retinopathy.

Advanced glycation end products (AGEs), formed by non-enzymatic reactions between reducing sugars and free amino groups of proteins or lipids that undergo complex and irreversible molecular rearrangement,5 accumulate in the retinal vasculature of patients with diabetes and streptozotocin-treated rats6,7 and have been implicated in the development of diabetic retinopathy.8,9 Chronic exposure of the endothelium to AGEs has been shown to increase retinal vascular permeability in vivo10 and in vitro.11 AGEs, however, have also been shown to increase capillary permeability acutely.12 Activation of the cell surface receptor for AGE (RAGE)13 and production of reactive oxygen species (ROS)14,15 have been shown to mediate cellular responses to AGEs; however, the signaling pathways involved in the early permeability response are unknown. In the present study we developed a novel technique, the isolated perfused retina, to study rapid changes in capillary permeability in response to agonists and have shown that the initial signaling events after application of AGEs requires RAGE and NADPH oxidase activation.

METHODS

Production of Glycated Albumin

AGE-modified albumin (AGE-BSA) was prepared from low endotoxin bovine plasma albumin (75 mg/mL) by incubation with D-glucose (0.5 M) in 0.5 M sodium phosphate buffer (pH 7.4 ± 0.05) containing 0.05% sodium azide at 37°C for 9 weeks. Nonglycated protein for control experiments was prepared under the same conditions except for the omission of glucose. N((carboxymethyl)lysine (CML) was prepared by incubating low endotoxin bovine plasma albumin (175 mg/mL) with glyoxylic acid (0.15 M) and sodium cyanoborohydride (0.45 M) in 0.2 M sodium phosphate buffer (pH 7.8 ± 0.05) at 37°C for 48 hours. Both types of AGE were dialyzed with PBS for 48 hours and sterile filtered. Endotoxin contamination was minimized by incubation of the samples with polymyxin B for 18 hours followed by centrifugation at 800g for 5 minutes. The supernatant was removed, and the endotoxin content was determined with the limulus amebocyte lysate assay (E-Toxate kit; Sigma, Poole, UK). Endotoxin content was less than 0.015 EU/mL in both solutions. CML content of glycated albumin produced by both routes was assessed by competitive ELISA with the use of a polyclonal anti-CML antibody obtained from rabbits immunized against AGE-BSA.10 CML content of the AGE-BSA preparation was approximately 200-fold higher than the nonglycated control, whereas the CML content of the CML-BSA preparation was approximately 2400-fold higher. Samples were freeze-dried and reconstituted in physiological buffer before experimentation.

Isolated Perfused Retina

Experiments were carried out in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research on the
excised retinas of male Wistar rats (weight range, 200–300 g), which were killed in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Immediately after death was confirmed, a common carotid artery was cannulated, and the vasculature was flushed with 9.0 g/L NaCl solution containing 300 U/mL heparin at room temperature until the fluid draining from the jugular vein was clear of blood. This was followed by perfusion of a stabilizing solution (10 mM Mg\(^{2+}\), 110 mM NaCl, 8 mM KCl, 10 mM HEPES, 1 mM CaCl\(_2\)) containing isoproterenol (10 μM) buffered to pH 7.0 ± 0.05 for 10 minutes, with Evans Blue-albumin (5.0 g/L Evans Blue dye, 10% albumin) added for the last minute. The eyeball was then enucleated, and the retina, still attached to the sclera, was dissected from the back of the eye and pinned out flat onto a transparent plastic column (Perspex; Dow-Corning, Midland, MI) capped with silicone encapsulant (Sylgard; Dow-Corning). The retina was continuously superfused with a Krebs solution containing 124 mM NaCl, 5 mM KCl, 2 mM MgSO\(_4\) (2 H\(_2\)O), 0.125 mM NaH\(_2\)PO\(_4\) (2 H\(_2\)O), 22 mM NaHCO\(_3\), 5 mM glucose, 2 mM CaCl\(_2\), and 0.1% BSA buffered to pH 7.4 ± 0.05, heated to 37°C ± 1°C, and gassed with 5% CO\(_2\) in air. The superfusate was delivered to the surface of the retina at a rate of 3 mL/min, giving a continuous bath volume of approximately 2 mL. A radial vessel (>20-μm diameter) on the surface of the exposed retina, observed to be well filled with Evans Blue-albumin, was cannulated with a glass micropipette filled with the low-molecular-weight fluorescent dye sulforhodamine B (1 mM) in Krebs solution, and the retinal microcirculation was filled by raising the pressure in the micropipette. The vasculature could be refilled if the fluorescent signal dissipated after several measurements were carried out. Extravasated dye was washed out of the interstitium by the superfusate fluid and did not rise appreciably during the course of an experiment. All chemicals were supplied by Sigma unless stated otherwise.

**Permeability Measurement**

The principle of the technique is essentially the same as described previously.\(^1\)\(^7\) Briefly, dye-filled small venules, 6 to 11 μm in diameter, identified by their tortuous nature and their location in the lower layer of the vascular bed, were selected for investigation. Vessels were imaged using a 40× water immersion (0.75 NA) objective (Zeiss, Jena, Germany) and were captured through a Leitz (Wetzlar, Germany) intravital microscope fitted with Leitz optics (Orthoplan) and fluorescence filters (Ploemopak; Leitz). A 530 ± 10-nm excitation filter was used to illuminate the retina, and images were recorded at more than 560 nm through a CCD camera (Intensified ISIS camera; Photonic Sciences, Robertsbridge, E. Sussex, UK) into a personal computer using a frame grabber (Matrox Pulsar; Dorval, QC, Canada) and were analyzed (ImageHopper; Samsara Research, Dorking, Surrey, UK). In these experiments, the pressure in the vasculature was allowed to equilibrate for 1 minute before images were captured. Estimates of permeability were obtained from a sequence of images taken at 2-second intervals over 100 seconds. The dye concentration difference across a vessel was estimated from the difference between cursors placed on an image stack (Fig. 1A). The signal from the cursor over the interstitium usually did not change during the experiment and represented autofluorescence rather than a buildup of dye lost from the vasculature. Previous experiments have shown that the light collected was linear with the dye concentration and with the square of the diameter of the vessel up to the limit of 60 μm.\(^1\)\(^8\) Permeability was determined from the rate of decrease of that difference, obtained by fitting an exponential to the data, such that

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P = \frac{k r}{2},
\]

where \(k\) is the rate constant and \(r\) is the vessel radius. (C) Basal permeability was recorded at 5-minute intervals and remained stable with a mean permeability of 0.31 ± 0.2 × 10\(^{-6}\) cm/s over 2 hours; \(n = 25\) vessels from 25 retinas. (D) Consistent increases in permeability were observed after application of AGE-BSA (350 μg/mL) at 10-minute intervals (\(n = 4\) vessels from four retinas).

**FIGURE 1.** Method of retinal microvascular permeability measurement. (A) Retinal vasculature was perfused with 1 mM sulforhodamine, and permeability measurement was obtained from the difference in dye concentration across a vessel wall (area under the cursors) from a sequence of images taken at 2-second intervals. (B) Permeability measurement was obtained from the rate of decrease of that difference obtained by fitting an exponential to the data, such that \(P = kr/2\), where \(k\) is the rate constant and \(r\) is the vessel radius. (C) Basal permeability was recorded at 5-minute intervals and remained stable with a mean permeability of 0.31 ± 0.2 × 10\(^{-6}\) cm/s over 2 hours; \(n = 25\) vessels from 25 retinas. (D) Consistent increases in permeability were observed after application of AGE-BSA (350 μg/mL) at 10-minute intervals (\(n = 4\) vessels from four retinas).
basal permeability remained steady (Fig. 1D). Subsequent experiments were carried out on venular capillaries, with initial resting permeability of less than 0.40 ± 10^-6 cm/s.

Culture of JG2.1 Retinal Endothelial Cells
JG2.1 rat-derived retinal endothelial cells (a kind gift from John Greenwood, Institute of Ophthalmology, University College London) were cultured at 37°C and 5% CO2 on collagen-coated plastic flasks in compounded nutrient mixture (Nutrient Mixture F-0; Gibco-BRL, Grand Island, NY) with L-glutamine, supplemented with 20% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.02 mg/mL endothelial cell growth factor.

Measurement of Fluorescence
Fluorescent ROS indicator (Amplex Red; Molecular Probes, Eugene, OR), in the presence of horseradish peroxidase, reacted with 1:1 stoichiometry with H2O2 to produce the highly fluorescent oxidation product resorufin, which could be measured with a spectrophotometer. JG2.1 retinal endothelial cells were seeded into the wells of black, clear-bottom, 96-well plates and were cultured in compounded nutrient mixture (Nutrient Mixture F-0; Gibco-BRL), as described, for 48 hours before treatment with 50 µL AGE-BSA. After incubation for 5 minutes with AGE-BSA, 50 µL working solution (100 µM fluorescent ROS indicator [Amplex Red; Molecular Probes] and 0.2 U/mL HRP in phosphate-buffered saline) was added to each well. Fluorescence was measured at 37°C immediately after the addition of working solution using a microplate reader (Hidex; Turku, Finland) with excitation and emission filters of 530 nm and 590 nm, respectively.

Statistical Analysis
Unless otherwise stated, results are expressed as the mean ± SE, and the significance of any changes was assessed by paired t-test. Regression lines and sigmoidal dose-response curves were fitted using GraphPad Prism version 4.02 for Windows.

RESULTS
AGE-BSA applied to the abluminal surfaces of retinal capillaries for 30 seconds resulted in a rapid and reversible dose-depen-
dent increase in permeability (Fig. 2A). The submaximal response at 350 μg/mL was used in subsequent experiments. Application of CML-BSA similarly resulted in a small, reversible, dose-dependent permeability increase that had characteristics similar to those of AGE-BSA (Fig. 2B). Nonglycated BSA, produced by both routes over the same range of concentrations, had no effect on permeability. Bradykinin also reversibly increased retinal capillary permeability after acute application. When anti-RAGE IgG was applied to the retina for 30 minutes, there was no alteration in basal permeability, but the result was a significant reduction of the response to AGE-BSA. The acute permeability response to CML-BSA was similarly reduced, but the response to bradykinin was unaffected (Fig. 2D), which is evidence that the RAGE receptor was involved in mediating the acute permeability response to AGE-modified albumin.

**Permeability Response to AGE-BSA Dependent on Reactive Oxygen Species**

The free radical scavengers superoxide dismutase (SOD) and catalase, in combination, reduced the permeability response to bradykinin and to AGE-BSA. Pretreating the preparation with the iron chelator, desferrioxamine, also significantly inhibited the permeability increase (Fig. 3). Similarly, coapplication of AGE-BSA with uric acid (200 μM) reduced the response (from 0.98 ± 0.1 to 0.04 ± 10⁻⁶ cm/s; P < 0.001; n = 6), as did pretreatment with butylated hydroxytoluene (10 μM) for 15 minutes, which reduced the response from 1.36 ± 0.2 to 0.23 ± 0.1 × 10⁻⁶ cm/s (P < 0.01; n = 4).

**Source of Free Radicals**

The permeability response to AGE-BSA was not significantly affected after pretreatment with the phospholipase A₂ (PLA₂) inhibitor palmitoyl trifluoromethyl ketone (PACOCF₃), but pretreatment with the flavoprotein inhibitor diphenylene iodonium (DPI) reduced the permeability increase, as did pretreatment with a low dose of apocynin (an NADPH oxidase inhibitor). Removing nitric oxide synthesis by applying Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) resulted in a significant increase in the permeability response. These data suggest that rapid activation of NADPH oxidase is responsible for the generation of ROS after activation of RAGE by AGE-modified albumin (Fig. 4).

**Direct Action of AGE-BSA on Endothelial Cells**

The previous data were obtained from abluminal application of AGE-BSA, a process by which many cell types could be responsible for the permeability response observed. Evidence that the endothelium was primarily responsible was obtained by perfusing the retinal vasculature with AGE-BSA. Permeability was unaffected as long as free radicals were removed by including SOD and catalase in the superfusing solution; however, when these were removed, capillary permeability rapidly increased. Removing SOD and catalase from the superfusing solution when the retinal vasculature was perfused with nonglycated BSA control had no effect on permeability (Fig. 5).

**Ca²⁺ and PKC Required for Permeability Response to AGE-BSA**

The permeability response to AGE-BSA was reduced when Ca²⁺ was removed from the superfusing and perfusing solutions. Coapplication of the divalent cation channel blocker SKF-96365 also inhibited the permeability increase, as did the phospholipase C (PLC) inhibitor U-73122. Pretreating the isolated retina with the protein kinase C (PKC) inhibitor calphostin C resulted in a significant reduction of the permeability response. Gö 6976, which selectively inhibits the Ca²⁺-depen-
dent PKC isoforms α and β1, also significantly reduced the permeability response to AGE-BSA. The permeability response to bradykinin was unaffected by PKC inhibitors and SKF-96365 (Fig. 6). These data suggest that activation of Ca²⁺-dependent isoforms of PKC are required for mediating the acute permeability response to AGE-BSA.

AGE-BSA Potentiation of the Acute Response to Bradykinin

The maximal permeability response to bradykinin was increased after 10-minute exposure to AGE-BSA, and log EC_{50} was reduced. Pretreatment of the retina with AGE-BSA increased the permeability response to bradykinin, which was sustained over a recording period of 20 minutes, with no effect on basal permeability. The potentiation of the bradykinin response was abolished when AGE-BSA was applied in the presence of apocynin (Fig. 7).

ROS Production Increase by AGE-BSA in Retinal Endothelial Cells

Treating JG2.1 retinal endothelial cells with AGE-BSA produced a dose-dependent increase in working solution fluorescence that was inhibited by pretreatment with anti-RAGE IgG, apocynin, calphostin C, and U-73122 (Fig. 8).

DISCUSSION

These experiments have shown that acutely applied CML albumin and the more heterogeneous AGE albumin, at concentrations found in patients with diabetes, resulted in dose-dependent and reversible increases in retinal microvascular permeability. This is, to our knowledge, the first direct measurement of permeability in retinal vessels and is in accordance with previous indications that AGEs are responsible for increased retinal vascular permeability.

Permeability values at rest were of an order similar to those of brain pial venules at approximately $3 \times 10^{-6}$ cm/s, which is equivalent to a transendothelial electrical resistance value of approximately 1200 Ωcm² and shows that these vessels are representative of the blood-retinal barrier. The experimental preparation remained stable for up to 3 hours, after which baseline permeability increased occasionally. Repeated application of the same dose of AGE-BSA at 10-minute intervals resulted in similar permeability increases, indicating that any RAGE downregulation had recovered within this period. Measurements were made on the most responsive vessels to provide optimal conditions for examining the signaling processes. This preparation is thus suited for studying the mechanisms underlying acute effects of inflammatory mediators rather than longer term ones. This has the advantage of giving...
A clear indication of the primary insult and the initial signaling pathways that can lead to secondary effects and new protein synthesis. Although AGE albumin was most commonly applied to the abluminal surfaces of the vessels and, hence, was in contact with other retinal cells, direct perfusion with AGE-BSA at a physiological concentration, was used in the superfusate; this has been shown to reduce the specificity of uric acid for peroxynitrite while enhancing its general ROS-scavenging properties. Free radical scavenging from the abluminal side was successful at reducing the permeability response to luminal AGE-BSA application because of superoxide escape across the endothelial cell membrane through chloride channels or via intracellular catalase conversion of superoxide to the more membrane-permeable hydrogen peroxide. It is possible that all interventions that reduce the effect of AGE-BSA may do so by directly altering this molecule rather than by affecting the signaling pathways involved in the response. We consider this to be unlikely because we used a variety of inhibitors that are molecularly dissimilar and because, in the experiment illustrated in Figure 5, abluminal application of the proteins superoxide dismutase and catalase inhibited the response to luminal applied AGE-BSA.

ROS generation has been shown to mediate rapid increases in permeability in pial venules after application of arachidonic acid or bradykinin, and retinal vessels appear to behave in a similar manner because the permeability response to bradykinin was also inhibited in the presence of SOD and catalase. In pial vessels bradykinin activates PLA2, resulting in the release of arachidonic acid, the metabolism of which, by cyclooxygenase and lipoxygenase, results in the formation of ROS. We found, however, that in the present experiments PACOCF3, which inhibits all PLA2 isoforms at the doses used, had no effect on the permeability response to AGE-BSA. Hence, other ROS-producing enzymes were involved in the acute permeability response.

Inhibiting eNOS with L-NAME did not reduce permeability, as would be expected if ROS were derived from uncoupled eNOS. L-NAME, however, potentiated the permeability response to AGE-BSA, possibly because of the reduced availability of NO to scavenge superoxide. Under physiological conditions, the peroxynitrite formed by this reaction should be rapidly metabolized by reduced glutathione; therefore, reducing NO production may lead to an apparent increase in ROS generation in response to stimulation.

Endothelial cells express a gp91phox-containing NADPH oxidase (NOX2) responsible for significant ROS production in response to agonist stimulation. Some evidence suggests that increased ROS generation by NADPH oxidase may play a significant role in the progression of diabetic microvascular complications. Pretreatment of the retina with DPI, a flavoprotein inhibitor, and apocynin (at a very low concentration), which prevents NADPH oxidase assembly, significantly reduced the permeability response to AGE-BSA, indicating that NADPH oxidase is responsible for the increased free radical production and subsequent permeability increase in these experiments. Apocynin also significantly reduced the permeability response to AGE-BSA, suggesting that NADPH oxidase is responsible for the increased free radical production and subsequent permeability increase in these experiments. Apocynin also significantly reduced the permeability response to AGE-BSA, suggesting that NADPH oxidase is responsible for the increased free radical production and subsequent permeability increase in these experiments.
reduced when Ca$^{2+}$/H10001 was removed from the perfusing and superfusing solutions or when the preparation was pretreated with SKF-96365, which inhibits Ca$^{2+}$/H10001 channels, or U-73122, which inhibits PLC. ROS production from cultured JG2.1 endothelial cells, after application of AGE-BSA, was also reduced when cells were treated with the PKC inhibitor calphostin C. It seems likely, therefore, that RAGE activation results in increased [Ca$^{2+}$/H10001]i through PLC, leading to the activation of PKC, which results in increased production of ROS from NADPH oxidase. PKC has been shown to phosphorylate tight junction proteins in the presence of proinflammatory mediators, thereby altering permeability.30 The present experiments, however, in which bradykinin was applied demonstrated that acute permeability responses may occur independently of PKC phosphorylation.

Preassembled NADPH oxidase may also be activated by the small monomeric G protein Rac.31 Bradykinin has been shown to activate Rac32 and, therefore, may itself activate NADPH oxidase once assembled and may provide an additional source of ROS within the endothelium. This was borne out by the present experiments in which the pretreatment of retinal vessels with AGE-BSA for 10 minutes potentiated the permeability response after stimulation with bradykinin; this potentiation was blocked in the presence of apocynin.

A number of different mechanisms have been suggested by which ROS may acutely increase microvascular permeability. Oxidative modification of Ca$^{2+}$/H10001 channels by ROS increases the release of Ca$^{2+}$/H10001 from intracellular stores,33 whereas the hydroxyl radical has also been shown to inhibit the endoplasmic reticulum ATPase.34 The consequent increase in intracellular Ca$^{2+}$/H10001 may lead to contraction of the cytoskeleton or the phosphorylation of tight junction proteins by PKC, thereby increasing permeability.35 On the other hand, conserved residues of protein phosphatases may be oxidized and inhibited by ROS.
causing increased phosphorylation of cytoskeletal or junctional proteins that may result in the disruption of cell contacts, causing permeability to increase.36

**FIGURE 7.** Effect of exposure to AGE on the permeability response to bradykinin. (A) Permeability response to acute bradykinin (1 µM) application was increased after 20 minutes of AGE-BSA (350 µg/mL; the permeability increase was $0.7 \pm 0.03 \times 10^{-6}$ cm/s before AGE and $1.9 \pm 0.19 \times 10^{-6}$ cm/s after AGE; $P < 0.01$; t-test; four vessels from four retinas). (B) Permeability response to bradykinin was significantly elevated for all doses, except the lowest (10 nM; two-way ANOVA; Bonferroni test), and the log EC$_{50}$ was reduced from $-6.1 \pm 0.15$ to $-7.0 \pm 0.18$ ($P < 0.05$; ANCOVA). (C) Permeability response to bradykinin (1 µM) was decreased when AGE was coapplied with apocynin (1 µM) (permeability before AGE apocynin $0.7 \pm 0.03 \times 10^{-6}$ cm/s and after $0.5 \pm 0.02 \times 10^{-6}$ cm/s; $P < 0.001$; four vessels from four retinas).

**FIGURE 8.** AGE-BSA increases ROS production in retinal endothelial cells. (A) AGE-BSA (3.5 µg/mL-3.5 mg/mL) produced a dose-dependent increase in the fluorescent ROS indicator when applied to confluent monolayers of JG2.1 retinal endothelial cells for 5 minutes, giving an log EC$_{50}$ of $-6.3 \pm 0.58$ (means of four cultures). (B) ROS production was attenuated after 1-hour pretreatment with anti–RAGE IgG (50 µg/mL) after 15-minute pretreatment with apocynin (100 µM), the PLC inhibitor U-73122 (10 µM), and the PKC inhibitor calphostin C (500 nM). Data from four cultures, ANOVA, Dunnett multiple comparison test.
It is likely that antioxidant defense mechanisms would be rapidly upregulated after the activation of NADPH oxidase, which may account for the transient nature of the response. Chronic activation of RAGE, as would occur within the retinas of patients with diabetes, would cause a long-term shift toward a prooxidant environment likely to be deleterious. Sustained production of ROS would be expected to cause retinal hyperpermeability because of the increased expression of vascular endothelial growth factor, possibly as a consequence of stabilization of hypoxia-inducible factor by ROS. The activation of proinflammatory transcription factors, such as NF-kB, may also be increased in a prooxidant environment, resulting in further increases in retinal microvascular permeability.

In conclusion, these experiments suggest that the ROS produced by rapidly activated NADPH oxidase increase retinal microvascular permeability that could be associated with the initial stages of macular edema and diabetic retinopathy. It is also likely that various stress response genes are activated to counter this. Nevertheless, there is likely to be an increased ROS load in response to agonists such as bradykinin that are capable of stimulating preassembled NOX2.

References


