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Involvement of NADPH oxidase and protein kinase C in endothelin-1-induced superoxide production in retinal microvessels

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ABSTRACT

Redox signaling has been implicated in pathophysiological changes in the vascular system. We examined whether endothelin-1 (ET-1) increases the formation of superoxide anions in retinal microvessels. Freshly isolated retinal microvessels from rats were exposed to ET-1 (100 nM), and the intracellular superoxide formation in the retinal pericytes was assessed semi-quantitatively by time-lapse fluorometric analyses using hydroethidine. The receptor mechanisms were determined by BQ-123 and BQ-788, receptor antagonists for ET_A and ET_B receptors, respectively, and also by IRL-1620, a selective agonist for ET_B receptors. In addition, the changes induced by adding apocynin (10 μM), myr-PKC (1.0 μM), allopurinol (100 μM), rotenone (10 μM), or L-NAME (100 μM) with ET-1 were evaluated. Microvessels were incubated with phorbol 12-myristate 13-acetate (PMA, 10 nM), a protein kinase C (PKC) activator. Fluorometric analyses showed ethidium fluorescence-positive regions that coincided well with the location of retinal pericytes. The intracellular superoxide levels were significantly increased after addition of ET-1 (100 nM), and this elevation was suppressed by apocynin or myr-PKC. Other enzyme inhibitors including L-NAME had no effect. The ET-1-induced increase of superoxide was significantly suppressed by BQ-123 (1.0 μM), while effects of adding BQ-788 (1.0 μM) were insignificant. IRL-1620 (100 nM) did not increase superoxide formation significantly. PMA (10 nM) mimicked the effect of ET-1. These results suggest that ET-1 increases the formation of superoxides in the retinal microvascular pericytes most likely by activating NADPH oxidase through ET_A receptors. The activation of PKC may be involved in the mechanism. Thus, ET-1 may augment its vasoconstrictive effects through the formation of superoxide, which may impair the bioavailability of nitric oxide in the retinal microvasculature.

1. Introduction

The retinal circulation is autoregulated to meet the local metabolic needs of the retina. One indication of the autoregulation is the absence of autonomic innervation (Ye et al., 1990). In addition, the retinal microvasculature lacks smooth muscle sphincters, which control capillary perfusion in other tissues (Pannarale et al., 1996). In the retina, it is thought that vasoactive molecules, such as the endothelins (ETs) and nitric oxide (NO) that are released from the vascular endothelium, neurons, and glia may play important roles in the regulation of retinal blood flow (Haefliger et al., 1994; Simard et al., 2003). In addition to the retinal arterioles, the control of retinal blood flow is regulated at the capillary level where the pericytes are considered to play a crucial role (Puro, 2007). In support of this, the ratio of pericytes to the endothelial cells is highest in the retinal vasculature (Shepro and Morel, 1993).

The ETs are a family of 21 amino acid peptides with three isomers, ET-1, ET-2, and ET-3. ET-1 is the most potent and long-acting vasoconstricting peptide of any investigated (Yanagisawa et al., 1988). ET-1 is expressed by retinal vascular endothelial cells (Takahashi et al., 1989), and pericytes have specific ET_A/ET_B receptors (Chakravarthy et al., 1992; McDonald et al., 1995). ET-1 increases the calcium levels in pericytes, causes pericytes to contract (Chakravarthy et al., 1992; Ramachandran et al., 1993), and constricts pericyte-containing retinal microvessels (Schonfelder et al., 1998).

Other important signaling molecules that modulate vascular tone include reactive oxygen species (ROS), e.g., superoxide and hydrogen peroxide (Lyle and Griendling, 2006). Superoxide inactivates nitric oxide (NO), and hydrogen peroxide, the dismutation form of superoxide, activates Rho/Rho kinase leading to vascular constriction (Jin et al., 2004).

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Evidence has been obtained by different techniques including gene-targeted molecular approaches that NADPH oxidase is the main vascular source of superoxide (Landmesser et al., 2002; Lassègue et al., 2001; Lavigne et al., 2001). Studies using pharmacological inhibitors have suggested that ET-1 increases superoxide production in the carotid artery in DOCA-salt hypertensive rats possibly through the activation of NADPH oxidase (Li et al., 2003). The ET-1-induced vasoconstriction is, in part, mediated by the production of ROS (Just et al., 2008). However, evidence that this mechanism plays a role in retinal microvessels is lacking. NADPH oxidase is expressed in retinal pericytes (Manea et al., 2005), and other potential sources of superoxides are xanthine oxidase (Jankov et al., 2008), NOS (Loomis et al., 2005), and mitochondria (Callera et al., 2003).

We hypothesized that ET-1 increases superoxide formation in retinal microvessels and that redox signaling is involved in the action of ET-1. To accomplish this, we investigated superoxide production by different pathways in pericyte-containing retinal microvessels of rats. Using fluorometric analyses and time-lapse recordings, we obtained evidence suggesting that the activation of ET_A receptors increased superoxide production in retinal microvessels by a mechanism involving NADPH oxidase. In addition, the activation of protein kinase C (PKC) may also be involved in the ET-1-induced increase of superoxide production.

2. Materials and methods

2.1. Animals

Nine-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in an air-conditioned room set to a temperature of approximately 23 °C and 60% humidity, and placed on a 12:12 light:dark cycle. They were used before they were 16 weeks of age. All the animals were handled in accordance with the ARVO resolution for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Committee of Animal Use and Care of the Osaka Medical College.

2.2. Chemicals

Unless noted, all the chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). ET-1 (Peptide Institute, Osaka, Japan) was dissolved in 0.1% acetic acid to make a 0.1 mM solution, and further dilutions were made in phosphate-buffered saline (PBS). BQ-123 and BQ-788 were used as ET_A and ET_B receptor antagonists, respectively. Apocynin was used as an inhibitor of NADPH oxidase. Other inhibitors used were: allopurinol, a xanthine oxidase inhibitor; rotenone, a mitochondrial electron transport chain complex inhibitor; myristoyl-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln (myr-ΨPKC), a PKC inhibitor; and L-NAME, a NOS inhibitor. We also used: phorbol 12-myristate 13-acetate (PMA), a PKC activator; IRL-1620, a selective agonist for ET_B receptors; and polyethylene glycol superoxide dismutase (PEG-SOD), a membrane-permeable SOD analog.

2.3. Microvessel isolation

Retinal microvessels were freshly isolated by a modified "tissue-print" method (Sakagami et al., 1999). For each experiment, retinas from a euthanatized rat were rapidly removed and incubated in 2.5 ml Earle's balanced salt solution (Life Technologies, Grand Island, NY) supplemented with (mM): 0.5 EDTA, 1.5 $CaCl_2$, 1 $MgSO_4$, 20 glucose, 26 sodium bicarbonate, 15 U papain (Worthington Biochemicals, Freehold, NJ), 0.04% DNase, and 2 mM cysteine for 30 min at 30 °C. The media was bubbled with 95% O_2–5% CO_2 for oxygenation and to maintain the pH. After the retinas were transferred to solution A (140 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Na-Hepes, 15 mM mannitol, and 5 mM glucose at pH 7.4 with osmolarity adjusted to 310 mosmol), they were cut into four parts. Then, each retinal quadrant was placed with its vitreal surface up in a glass-bottomed chamber that contained 1 mL of solution A. Each retinal quadrant was sandwiched between the bottom of the chamber and a 15 mm diameter glass cover slip that was gently placed on the vitreal surface of the retina. When the cover slip was removed, the vessels adhered to the cover slip.

It has been demonstrated that pericytes in the proximal and distal portions of the retinal microvessels have different properties (Matsushita and Puro, 2006). We assessed the proximal portion of the pericyte-containing retinal microvessels, which were located distal to the smooth muscle-encircled arterioles and proximal to the capillaries (Fig. 1A). The density of pericytes in the proximal portion has been suggested to be >4/100 μm segment, and the proximal portion of retinal microvessels could be distinguished from the distal part by their density (Matsushita and Puro, 2006). Experiments were performed within 3 h after the isolation of the retinal vessels, and 2–3 microvessels/rat could be monitored by time-lapse recordings. We found that the mean density (±SD) of pericytes in our proximal vessels was 4.8 ± 0.7/100 μm segment.

2.4. Detection of intracellular superoxide production by time-lapse recordings

The level of superoxide within the pericytes of the retinal microvessels was measured by hydroethidine, a specific fluorogenic probe. Hydroethidine is oxidized by superoxide to ethidium, a fluorescent product, which is retained intracellularly and allows a semi-quantitative estimation of the cellular superoxide level (Bindokas et al., 1996; Zhao et al., 2005).

Time-lapse photographs were taken to determine the early changes in the superoxide level induced by exposure to ET-1. Thirty

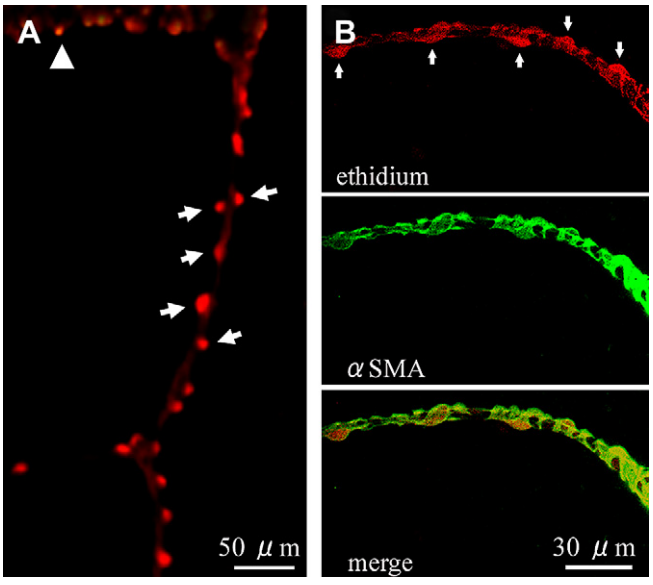

Fig. 1. A) Expression of ethidium fluorescence in retinal microvessels. Photograph is taken just distal to the vascular smooth muscle-enriched arteriole (arrow head) by a fluorescence microscope. Strong ethidium fluorescent regions (arrows) can be seen on the ablumenal wall, and these locations are characteristic to those of pericytes. (B) Co-localization of ethidium fluorescence and pericytes labeled by FITC-conjugated αSMA. Strong ethidium-positive regions (arrows) well coincide with the somas of pericytes. Thus, ethidium fluorescence assay is considered to reflect the superoxide formation in the retinal pericytes.