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Reactive oxygen species facilitate the EDH response in arterioles by potentiating intracellular endothelial Ca^{2+} release

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ABSTRACT

There is abundant evidence that H_2O_2 can act as an endothelium-derived hyperpolarizing factor in the resistance vasculature. However, whilst scavenging H_2O_2 can abolish endothelial dependent hyperpolarization (EDH) and the associated vascular relaxation in some arteries, EDH-dependent vasorelaxation can often be mimicked only by using relatively high concentrations of H_2O_2. We have examined the role of H_2O_2 in EDH-dependent vasodilatation by simultaneously measuring vascular diameter and changes in endothelial cell (EC) $[Ca^{2+}]_i$ during the application of H_2O_2 or carbachol, which triggers EDH. Carbachol (10 μM) induced dilatation of phenylephrine-preconstricted rat cremaster arterioles was largely (73%) preserved in the presence of indomethacin (3 μM) and L-NAME (300 μM). This residual NO- and prostacyclin-independent dilatation was reduced by 89% upon addition of apamin (0.5 μM) and TRAM-34 (10 μM), and by 74% when an extracellular ROS scavenging mixture of SOD and catalase (S&C; 100 U ml^{-1} each) was present. S&C also reduced the carbachol-induced EC $[Ca^{2+}]_i$ increase by 74%. When applied in Ca^{2+}-free external medium, carbachol caused a transient increase in EC $[Ca^{2+}]_i$. This was reduced by catalase, and was enhanced when 1 μM H_2O_2 was present in the bath. H_2O_2 -induced dilatation, which occurred only at concentrations $\geq$ 100 μM, was reduced by a blocking antibody to TRPM2, which had no effect on carbachol-induced responses. Similarly, iberotoxin and Rp-8bromo cGMP reduced the vasodilatation induced by H_2O_2, but not by carbachol. Inhibiting PLC, PLA_2 or CYP450 2C9 each greatly reduced the carbachol-induced increase in EC $[Ca^{2+}]_i$ and vasodilatation, but adding 10 μM H_2O_2 during PLA_2 or CYP450 2C9 inhibition completely restored both responses. The nature of the effective ROS species was investigated by using Fe^{2+} chelators to block the formation of •OH. A cell permeant chelator was able to inhibit EC Ca^{2+} store release, but cell impermeant chelators reduced both the vasodilatation and EC Ca^{2+} influx, implying that •OH is required for these responses. The results indicate that rather than mediating EDH by acting directly on smooth muscle, H_2O_2 promotes EDH by acting within EC to enhance Ca^{2+} release.

1. Introduction

Endothelium-dependent hyperpolarization (EDH) plays an important role in the vasodilatation of small arteries and arterioles induced by diverse stimuli including locally released factors, blood flow, and low pH [1]. EDH was initially viewed as resulting from the release of a diffusible endothelial factor, termed EDHF [2], but is now also thought to involve the transmission of endothelial cell hyperpolarization to the underlying vascular smooth muscle cells (VSMCs) *via* myoendothelial gap junctions [3]; the relative importance of each mechanism is thought to vary between different arteries/arterioles and also depends on the degree of pre-existing vascular excitation [4]. In either case, calcium-activated potassium channels $K_{Ca}2.3$ and $K_{Ca}3.1$ (SK_{Ca} and IK_{Ca} respectively) have been shown to play a significant part in the EDH response (EDHR).

Pomposiello et al. [5] suggested that H_2O_2 has a role to play in

Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; AUDA, 12-(3-adamantan-1-yl-ureido)dodecanoic acid; CP85, Na-1-ethylsulfoxide-2-methyl-3-hyroxypyridin-4-one; CP94, 1,2-diethyl-3-hyroxypyridin-4-one; CPA, Cyclopiazonic Acid; DFO, Desferrioxamine; EC, endothelial cell; EDH, endothelial dependent hyperpolarization; EDHF, EDH factor; EDHR, EDH response; EETs, epoxyeicosatrienoic acids; EEZE, 4, 15-epoxyeicosa-5(Z)-enoic acid; sEH, epoxide hydrolase; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBTx, Iberiotoxin; IL-1β, interleukin-1β; IP_3R, IP_3 receptor; L-NAME, N(G)-nitro-L-arginine methyl ester; L-NNA, Nω-Nitro-L-arginine; NO, nitric oxide; ODYA, 17-octadecynoic acid; PE, phenylephrine; PGI_2, prostacyclin; PKG, protein kinase G; PLA_2, phospholipase A_2; $cPLA_2$, cytoplasmic PLA_2; PLC, phospholipase C; PPOH, 2-(2-propynyloxy)-benzenehexanoic acid; PSS, physiological salt solution; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SOD, superoxide dismutase; S&C, SOD and catalase; VSMC, vascular smooth muscle cell

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the EDHR, and a little later it was shown that L-NNA- and indomethacin-resistant vasodilatation and hyperpolarization elicited by acetylcholine in mouse small mesenteric arteries was attenuated by catalase and associated with increased endothelial ROS production [6]. Further evidence that H_2O_2 acts as an EDHF was also reported for dog coronary resistance arteries [7,8] and human small mesenteric [9] and coronary arteries [10]. Liu et al. [11] showed that an endothelium-denuded segment of human coronary artery dilated when it was perfused by the effluent from an upstream endothelium-intact artery subjected to increased shear stress, and that this dilatation was attenuated if an H_2O_2-scavenging column was interposed between the donor and recipient arteries. The possibility that H_2O_2 acts as an EDHF is also consistent with observations by other groups that application of exogenous H_2O_2 opens VSMC BK_{Ca} channels [12], hyperpolarizes VSMCs [13] and relaxes a variety of vascular preparations [10,14–16]. The evidence for H_2O_2 as the primary contributor to the EDHR evoked by bradykinin is also compelling for cerebral arterioles, in which it appears that cyclooxygenase-dependent production of H_2O_2 causes vasodilation by acting on BK_{Ca} channels [16,17]. Most convincingly, Eaton et al. [18] showed that a mouse with knocked-in redox-insensitive protein kinase G were hypertensive and had mesenteric vessels that were less sensitive to applied H_2O_2. However, one concern with many of the studies in which the effect of exogenous H_2O_2 was examined is that the EC_{50} for H_2O_2 – induced vasodilatation of endothelium-denuded arteries was above 10 μM *e.g.* [10,14,16] Notably, Liu et al. [11] reported that whereas the vasodilating effluent they collected from endothelium-intact perfused human coronary arteries contained 0.6 μM H_2O_2, vasodilatation of endothelium-denuded arteries by exogenous H_2O_2 was negligible at a concentration of 10 μM.

Intriguingly, although H_2O_2 dilated endothelium-intact human submucosal intestinal microvessels, it caused constriction following endothelial denudation, a finding which led to the suggestion that H_2O_2 promotes the release of a separate EDHF instead of itself acting as an EDHF [15]. It was subsequently shown by Griffith and co-workers that the application of 100 μM H_2O_2, or the oxidizing agent thimerosal, to rabbit aortic valve endothelial cells (EC) enhanced intracellular Ca^{2+} release evoked by the SERCA inhibitor cyclopiazonic acid (CPA). Moreover, CPA-induced vasodilatation of rabbit ileac arteries was attenuated by application of catalase. They proposed that H_2O_2 could potentiate EDH-dependent vasodilatation by increasing IP_3 - dependent Ca^{2+} release [19]. Soon afterwards this group demonstrated that applying H_2O_2 concentrations of 10–30 μM also potentiated the EDH-associated vasodilation caused by acetylcholine in rabbit ileac arteries [20].

These studies suggest strongly that H_2O_2 acts to enhance rather than mediate the EDHR, and pose a number of important questions which are currently unanswered. As the ability of H_2O_2 to facilitate the EDHR has not been compared to its direct vasodilator effects in any single preparation, it remains unknown which of these effects are likely to be of predominant importance during the EDHR. Also, the pathways leading to H_2O_2 production within EC which would facilitate increases in EC $[Ca^{2+}]_i$ during the EDH response have not been defined. In addition, it is not clear whether physiological stimuli which have been shown to increase ROS production in EC lead to increases in the EDHR as would be predicted by this mechanism.

We have investigated these issues in arterioles in the intact rat cremaster circulation, as this preparation exhibits robust, sustained and consistent dilatations to both carbachol and H_2O_2, and permits endothelial Ca^{2+} concentration changes to be measured simultaneously. We characterized the effects of anti-oxidants and blockers of potential ROS-producing pathways on increases in EC $[Ca^{2+}]_i$ and the associated NO- and PGI_2-independent vasodilation evoked by the muscarinic receptor agonist carbachol. In addition, we carried out a comparison of the effects of carbachol and exogenously applied H_2O_2 in order to determine whether H_2O_2 might be acting as an EDHF. We also assessed whether EDH and ROS contribute to the vasodilatation induced by the inflammatory mediator cytokine IL-1β. Our results show that under basal conditions, endogenous ROS production involving CYP92C, phospholipase A_2 and hydroxyl radical greatly facilitates EC Ca^{2+} release and EDH-associated vasodilation. A brief pre-incubation with IL-1β further enhances the responses to carbachol. Although H_2O_2 also dilated these arterioles directly, it only did so at high concentrations, and *via* mechanisms which differ markedly from those evoked by carbachol.

2. Methods

This study conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and is in accordance with UK Home Office regulations (Animals Scientific Procedures) Act, 1986.

4–6 week old male Wistar rats (Charles River, UK) weighing 80–100 g were killed by exposure to an increasing concentration of CO_2 followed by cervical dislocation. A midline incision was made along the abdomen and the skin and muscle layer were retracted to expose the underlying organs. The inferior vena cava was tied off above the bifurcation into the common iliac veins and below that an incision was made to allow blood to drain from the lower extremities. A 0.61 mm fine bore polythene cannula was inserted down to the bifurcation into the common iliac arteries *via* the abdominal aorta and secured with thread. The left common iliac and the right femoral and internal iliac arteries were ligated to ensure that perfusion was only to the right external iliac artery supplying the cremaster artery and the cremaster muscle. This was then perfused through the cannula with a stabilizing solution (10 Mg^{2+}, 110 NaCl, 8 KCl, 10 HEPES, 1 $CaCl^{2+}$ all mM) containing heparin (30 U/ml) with isoproterenol 10 μM buffered to pH 7.0 ± 0.05 for 10 min to remove all blood.

The cremaster muscle was prepared by a midline incision along the scrotum to expose the right testes. Skin and connective tissue were carefully removed from the underlying cremaster muscle, which was then cut longitudinally along the anterior surface from the apex to the inguinal canal while keeping the vascular supply and drainage at the base intact. The testis was separated from the cremaster muscle and was pulled through the inguinal canal so as not to obstruct the preparation. The muscle was spread out flat onto a clear Sylgard disc using forceps and secured using microhistology pins, and the cardioplegic solution perfusing the circulation was then replaced for the duration of the experiment with PSS containing 0.1% albumin delivered by a gravity controlled reservoir. The preparation was moved to the stage of a Leitz Intravital microscope equipped with Orthoplan optics, and was superfused with PSS at 37 °C at a flow rate of 2.5 ml/min. PSS contained the Na^+ channel blocker lidocaine (20 mg l^{-1}) to block neural activity and keep the cremaster muscle from contracting.

Following a 30 min equilibration period an appropriate 2nd order arteriole (diameter 50–100 μm) was constricted by superfusion with 30 μM phenylephrine (PE) in combination with 300 μM N(G)-nitro-L-arginine methyl (L-NAME) and 3 μM indomethacin to inhibit NO and PGI_2 mediated vasodilatation. Constriction was maintained for 20 min to ensure its stability, and the muscarinic receptor agonist carbachol (10 μM) was then added for 2 min to the superfusate to elicit vasodilation. Arterioles were used for experiments only if a vasodilatation of > 50% (measured as described below) was observed.

During the experiments PE, L-NAME and indomethacin were present throughout. Drugs used to characterize the EDHR were