NADPH-INDUCED CONTRACTIONS OF MOUSE AORTA DO NOT INVOLVE NADPH OXIDASE: A ROLE FOR P2X RECEPTORS

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ABSTRACT

Reactive oxygen species (ROS) elicit vascular effects ranging from acute dilatation due to hydrogen peroxide-mediated opening of K^+ channels, to contraction arising from superoxide-dependent inactivation of endothelium-derived nitric oxide. Given that NADPH oxidases are major sources of superoxide in the vascular wall, this study examined the effects of exogenous NADPH, a substrate of these enzymes, on superoxide generation and isometric tone in mouse isolated aortic rings. NADPH caused concentration-dependent increases in superoxide generation (measured by lucigenin-enhanced chemiluminescence) and vascular tone (isometric tension recordings). However, surprisingly, while oxidised $NADP^+$ was unable to support superoxide production, it was equally as effective as reduced NADPH at stimulating vasocontraction. Also, an NADPH oxidase inhibitor, diphenyleneiodonium, markedly attenuated NADPH-induced superoxide production, yet had no effect on vasocontractions to NADPH. In contrast, a broad specificity P2X receptor antagonist, PPADS, as well as the P2X1 selective antagonist, NF023, markedly attenuated both endothelium-dependent and -independent vasocontractions to NADPH, as did the P2X desensitising agent, α,β -methylene-ATP. Importantly, α,β -methylene-ATP had no effect on superoxide production induced by NADPH. In conclusion, these findings suggest little role for NADPH oxidase-derived superoxide in the contractile effects of NADPH in the mouse aorta. Rather, NADPH appears to act as an agonist at two distinct P2X receptor populations; one located on the endothelium and the other on smooth muscle layer, both of which ultimately lead to contraction.

INTRODUCTION

NADPH oxidases are a major source of superoxide and downstream reactive oxygen species (ROS) in the vasculature and upregulation of these enzymes is a major cause of the oxidative stress associated with vascular diseases (Bengtsson et al., 2003; Madamanchi et al., 2005). NADPH oxidases generate superoxide by transferring electrons from the pyridine nucleotide, NADPH [and possibly NADH; see (Lassegue and Clempus, 2003)] to molecular oxygen. These enzymes were first identified in neutrophils as the enzyme complex responsible for the respiratory burst; however, recently isoforms of NADPH oxidase have been identified in non-phagocytic cells, including all major cell types that make up the blood vessel wall (i.e. endothelial and smooth muscle cells and adventitial fibroblasts) (Lassegue and Clempus, 2003). At least three isoforms of NADPH oxidase have been identified in the vascular wall, all of which are built on the same basic molecular plan consisting of cytosolic organiser (p47phox or NoxO1) and activator (p67phox or NoxA1) subunits, a small G-protein, (Rac1 or Rac2) and a membrane-bound cytochrome b558 reductase domain (Lassegue and Clempus, 2003). Cytochrome b558 is actually a heterodimer made up of a small α -subunit, p22phox, and a larger catalytic β -subunit or Nox, the nature of which varies with different isoforms of the enzyme (Lassegue and Clempus, 2003). Within vascular cells NADPH oxidase isoforms containing Nox1, Nox2 (gp91phox) and Nox4 have been identified (Bengtsson et al., 2003; Lassegue and Clempus, 2003).

In contrast to the NADPH oxidase expressed in phagocytes, which only becomes activated following exposure of cells to pathogens or pro-inflammatory mediators, a large pool of vascular NADPH oxidase is constitutively assembled (Li and Shah, 2002). This characteristic of the vascular enzyme means that addition of an excess of the pyridine nucleotide substrate, NADPH (or NADH) alone to vascular preparations is sufficient to increase superoxide

production (Li and Shah, 2002). Interestingly, NADPH appears to be equally effective at driving superoxide production whether added to intact or permeabilised preparations (Pagano et al., 1995; Li and Shah, 2001; Souza et al., 2001; Ellmark et al., 2005) indicating either that extracellular NAD(P)H gains entry to the intracellular compartment by an undefined mechanism or that the NAD(P)H binding site of vascular NADPH oxidase is accessible from the extracellular surface of the plasma membrane.

ROS are known to elicit a range of effects on vascular tone ranging from H₂O₂-mediated opening of K⁺ channels and subsequent vasodilatation, to vasoconstriction arising from superoxide-dependent inactivation of nitric oxide (NO). Hence, a number of recent studies have examined the vasomotor effects of NADPH and NADH in vitro and in vivo in order to assess the role of NADPH oxidase in regulation of vascular tone (Souza et al., 2001; Didion and Faraci, 2002; Paravicini et al., 2004; Park et al., 2004). However, in addition to augmenting NADPH oxidase activity, pyridine nucleotides could conceivably modulate vascular tone via stimulation of P2 nucleotide receptors. Indeed, many blood vessels are known to express nucleotide receptors both on the endothelial and smooth muscle layers (Kunapuli and Daniel, 1998). Moreover, in non-vascular smooth muscle preparations including the guinea pig taenia coli and rat anococcygeus muscle, responses to NADPH and NADH were due to activation of P2 and A1 receptors, respectively (Burnstock and Hoyle, 1985; Najbar et al., 1994). Of note, there were no qualitative differences between the oxidised [NAD(P)⁺; electron depleted] and reduced forms of each nucleotide in terms of their ability to modulate smooth muscle tone (Najbar et al., 1994), providing further evidence for a non-NADPH oxidase-dependent action.

In the present study, we examined the relative contributions of NADPH-dependent oxidases (e.g. NADPH oxidase and nitric oxide synthase) and P2 nucleotide receptors to vascular responses (superoxide production and vasocontraction) elicited by NADPH in mouse isolated

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thoracic aortas for the following two reasons: (1) the mouse aorta is known to express a functional NADPH oxidase and addition of NADPH to intact rings has previously been shown increase both superoxide production and vascular tone (Souza et al., 2001); and (2) P2 receptors were recently established to be expressed on both the endothelium and vascular smooth muscle cell layers of the mouse aorta and these receptors could conceivably contribute to the established vasoconstrictor effects of NADPH in this tissue (Beny, 2004). Our findings confirm previous observations that NADPH increases superoxide production from NADPH oxidase in the mouse aorta. However, this action does not contribute to the vasoconstrictor effects of NADPH in this tissue. Rather, activation of P2X receptors on both the endothelium and smooth muscle layers is the predominant mechanism by which NADPH modulates aortic tone.

METHODS

Mice

12-15 week-old male C57BL6/J mice, maintained on a normal chow diet and 12 hour day/night cycle, were used in this study with the approval of the Monash University Department of Pharmacology and Howard Florey Institute animal ethics committees. For all experiments, animals were heparinised (500 IU, i.p.; DBL, Australia) and anaesthetised with isoflurane (Rhodia, Australia) prior to being killed by decapitation. The entire aorta was excised, divided into the thoracic segment for lucigenin-enhanced chemiluminescence and the abdominal segment for isometric tension assays. Note in pilot experiments we found no differences between thoracic and abdominal aortic segments in terms of their ability to produce superoxide in response to NADPH.

Lucigenin-enhanced chemiluminescence

Following isolation, the thoracic aorta was immediately placed in ice-cold Krebs-HEPES buffer containing (in mM) NaCl 99, KCl 4.7, KH_2PO_4 1.0, MgSO_4 1.2, CaCl_2 2.5, NaHCO_3 25, Na-HEPES 20 & glucose 11 (pH 7.4). Aortas were cleared of adherent fat and cut into 2mm rings. In some rings the endothelium was removed by gently abrading the luminal surface of the vessel with a stainless steel pin. Vascular superoxide production was measured by 5 μM lucigenin-enhanced chemiluminescence as described previously (Paravicini et al., 2002). Briefly, aortic rings were pre-incubated for 45 mins at 37°C in Krebs-HEPES buffer containing either no further additives or one or more of the following drugs: diethyldithiocarbamic acid (DETCA; 3mM; to inactivate endogenous SOD1 and SOD3 activity); NADPH (1 - 3000 μM); NADH (100 μM); NADP^+ (100 μM); diphenyleneiodonium (5 μM); apocynin (1mM); native $\text{Cu}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase (SOD1; 300 Units/mL); native Mn^{2+} -superoxide dismutase (SOD2; 60 Units/mL); M40403 (30 μM); tiron (10mM); N^G -nitro-L-arginine methyl ester (L-NAME; 100 μM);

indomethacin (3 μ M); allopurinol (100 μ M); rotenone (1 μ M); α,β -methylene-ATP (10 μ M). Aortic rings were then transferred into the wells of an opaque white 96-well plate, each of which contained 300 μ L of a Krebs-HEPES-based assay solution consisting of 5 μ M lucigenin as well as the appropriate substrate/inhibitor compound(s). The plate was then placed into a TopCount Microplate Scintillation/Luminescence Counter (Packard, USA) and tissue-dependent photon emission per second per well was monitored over a 20-minute period. At the completion of the assay, aortic rings were dried in a 60°C oven for 24h enabling superoxide production to be normalised to dry tissue weight.

Isometric tension assay

Abdominal aortas were placed into ice cold Krebs-bicarbonate buffer [composition in mM: NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25 & glucose 11 (equilibrated with 95% O₂/5% CO₂)] and cut into four 2mm ring segments. Rings were then placed into the chambers of a myograph (Model 610M, Danish Myo Technology A/S, Denmark) with cold Krebs-bicarbonate buffer and gradually warmed to 37°C before being mounted on two stainless steel wire callipers (100 μ m diameter), one connected to a force displacement transducer and the other to a micrometer. Rings were then incubated under zero tension for 20 mins, after which time the baseline tension was elevated to 5mN by manually adjusting the micrometer. After a further 30 mins, maximal contractions of the tissue (U_{max}) was determined using U46619 (0.3 μ M, 15 mins). Aortic rings were then washed with fresh Krebs (x3) and allowed to return to baseline tension (~40 mins). Some rings were then treated for 20 minutes with one of the following inhibitor drugs: tiron (10mM); diphenyleneiodonium (5 μ M); L-NAME (100 μ M); α,β -methylene-ATP (10 μ M); PPADS (100 μ M); NF023 (10 μ M). Tissues were then precontracted to between 45 and 55 % U_{max} by titrating the concentration of U46619 in the organ chamber. Once a stable

plateau of tone was attained, cumulative half log molar concentrations of either NADPH, NADP^+ or NADH (1 – 300 μM) were added. The experiment was completed by addition of Ach (30 μM) to confirm the presence / absence of a functional endothelium.

Drugs

Acetylcholine chloride (Ach), allopurinol, α,β -Methylene-adenosine 5'-triphosphate lithium salt (α,β -methylene-ATP), β -nicotinamide adenine dinucleotide phosphate oxidised sodium salt (NADP^+), β -nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), β -nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), $\text{Cu}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase from bovine erythrocytes (SOD1), 9,11-Dideoxy-11 α ,9 α -epoxymethanoprostaglandin $\text{F}_{2\alpha}$ (U46619), 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt (tiron), diphenyleneiodonium chloride, 4'-hydroxy-3'-methoxyacetophenone (apocynin), indomethacin, NF023, N^G -nitro-L-arginine methyl ester hydrochloride (L-NAME), N,N'-dimethyl-9,9'-biacridinium dinitrate (lucigenin), sodium diethyldithiocarbamic acid trihydrate (DETCA), rotenone and pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (PPADS) were all purchased from Sigma-Aldrich (USA). M40403 [a manganese(II)-bis(cyclohexylpyridine)-substituted macrocyclic superoxide dismutase mimetic] was obtained from Metaphor Pharmaceuticals (USA).

Data analysis

Superoxide production was expressed either as relative light units per second per mg of dry tissue weight (Figure 1A) or as a percentage of the median control value of each experimental group. U_{max} values are expressed in mN, while changes in isometric tension to NADPH, NADP^+ and NADH are expressed as a percentage of the U46619-induced precontraction. All results are expressed as mean $\pm$ standard error of the mean (S.E.M.) of experiments conducted on tissues

taken from n animals. Concentration-dependent response curves for NADPH, NADP⁺ and NADH were computer-fitted (Graphpad Prism, Version 4.00) with a sigmoidal regression curve of equation:

$$Y = \text{BOTTOM} + (\text{TOP} - \text{BOTTOM}) / (1 + 10^{(\text{pEC}_{50} - X) \cdot \text{HILLSLOPE}}) \quad (\text{Eq. 1})$$

where X is the logarithm of the agonist concentration, Y is the response (i.e. superoxide production or vasocontraction), BOTTOM is the lower response plateau, TOP is the upper response plateau and pEC₅₀ is the logarithm of the X value when the response is half-way between BOTTOM and TOP. The variable HILLSLOPE controls the slope of the curve. Statistical comparisons were made using either Student's t-test or one-way or two-way ANOVA with Tukey-Kramer's posthoc test. A value of P<0.05 was considered statistically significant.

RESULTS

Vasocontractions to NADPH are endothelium- and superoxide-dependent

Lucigenin-enhanced chemiluminescence was barely detectable in mouse isolated aortic rings under basal conditions (Figure 1A). However, exposure to exogenous NADPH caused a concentration-dependent increase in chemiluminescence (pEC_{50} , 3.7 ± 0.3 ; R_{max} , 11443 ± 1521 RLU/s/mg), which was further enhanced in the presence of the SOD inactivating agent, DETCA (pEC_{50} , 4.0 ± 0.1 ; R_{max} , 51081 ± 5454 RLU/s/mg; $P < 0.01$; Figure 1A). NADPH-dependent chemiluminescence was markedly inhibited by the cell permeable superoxide scavenging compound, tiron, confirming that the assay was specific for superoxide, whereas cell impermeable SOD2 had no effect (Figure 1B). Likewise, a structurally unrelated superoxide scavenger, M40403, inhibited chemiluminescence, while SOD1 had no effect (see Online Data Supplement - Figure 1). Also, NADPH-dependent superoxide production was augmented by ~50% by mechanical removal of the endothelium (Figure 1C).

In isometric tension studies, NADPH caused concentration-dependent contractions in rings of aorta precontracted with U46619 (Figures 2A and B). NADPH elicited a similar effect on vascular tone in rings of artery precontracted with phenylephrine but had no effect in rings maintained under resting tension (see Online Data Supplement - Figure 2). Removal of the endothelium inhibited contractions to NADPH by ~50% (Figure 2B) and relaxations to Ach by ~80% (Figure 2C). Interestingly, tiron had no significant effect on contractions to NADPH in endothelium-intact tissues but markedly attenuated the response in endothelium-denuded tissues (Figure 2A). Tiron had no effect on responses to Ach in endothelium-intact or -denuded tissues (Figure 2B).

Vasocontractions to NADPH occur independently of NADPH oxidase and eNOS activity.

Whereas reduced NADPH stimulated a large increase in superoxide production, its oxidation product, NADP^+ , which lacks a donatable hydride ion (electron) and thus the ability to support enzymatic reduction of molecular oxygen, failed to induce superoxide production above basal levels (Figure 3A). NADH, which has been mooted as an alternative/preferred substrate for some isoforms of NADPH oxidase (Lassegue and Clempus, 2003), also had no significant effect on superoxide levels (Figure 3A). Surprisingly, despite its inability to stimulate superoxide production, NADP^+ was equally as effective as NADPH at eliciting contractions in precontracted mouse aortic rings (Figure 3B). In contrast, NADH induced a small relaxation response (Figure 3B).

The above findings indicate that the effect of NADPH on aortic tone may not always be related to its ability to augment superoxide production from NADPH-dependent vascular oxidases such as NADPH oxidase. To further explore this possibility we compared the effects of known inhibitors of NADPH oxidase and other potential sources of vascular ROS, on superoxide production and contraction. NADPH-dependent superoxide production was significantly reduced after acute exposure of aortic rings to the flavin antagonist and non-selective NADPH oxidase inhibitor, diphenyleneiodonium ($P < 0.001$, $n = 6$; Figure 4A). Consistent with our previous observation in mouse cultured vascular smooth muscle cells (Ellmark et al., 2005), short term incubation with apocynin failed to reduce NADPH-dependent superoxide production (Figure 4A). In contrast, when the exposure time to apocynin was increased to 24h, NADPH-stimulated superoxide production appeared to be markedly attenuated (Figure 4B); however, this effect just failed to reach statistical significance due to the large variation in control responses to NADPH in aortic rings after 24h of organoid culture. L-NAME, an inhibitor of nitric oxide synthase, also appeared to reduce NADPH-dependent superoxide production by ~40%, but again this just failed

to reach significance, while inhibitors of cyclooxygenase (indomethacin), xanthine oxidase (allopurinol), and the mitochondrial electron transport chain (rotenone), had no effect (Figure 4C). These results suggest that NADPH oxidase (and possibly eNOS) contribute to vascular superoxide production in response to NADPH. However, neither diphenyleneiodonium nor L-NAME had any effect on the contractile response to NADPH in isolated aortic rings (Figure 5A) despite both treatments abolishing endothelium-dependent relaxations to Ach (Figure 5B).

Vasocontractions to NADPH are mediated by P2X receptors.

NADPH has previously been described as an agonist of P₂ receptors (Burnstock and Hoyle, 1985; Najbar et al., 1994). Therefore, we examined the influence of α,β -methylene-ATP (a P2X desensitising agent) on NADPH-induced vasocontractions in mouse isolated aorta. α,β -methylene-ATP caused large contractions, which were similar in magnitude in endothelium-intact and -denuded rings of aorta. In both cases, responses to α,β -methylene-ATP spontaneously returned to baseline levels of tension after 3 minutes, presumably reflecting desensitisation of the P2X effector pathways that lead to vasocontraction in this tissue. Pretreatment with α,β -methylene-ATP had no effect on the concentration of U46619 required to precontract tissues to ~50% U_{max} but markedly attenuated responses to NADPH in both endothelium-intact and denuded tissues (Figure 6A). In contrast, α,β -methylene-ATP had no effect on NADPH-induced superoxide production in mouse aortic rings (Figure 6B), further highlighting the fact that NADPH induces vasocontraction and superoxide production via different mechanisms.

To confirm the involvement of P2X receptors in vasocontractions to NADPH, we examined the effects of PPADS, a broad selectivity P2X receptor antagonist, and NF023, which is a relatively selective P2X₁ antagonist. Vasocontractions to NADPH in both endothelium-denuded and -intact tissues were virtually abolished in the presence of PPADS (Figure 7A).

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NF023 also inhibited vasocontractions to NADPH. In both endothelium-intact tissues NF023 suppressed responses to NADPH by ~50%. In endothelium-denuded tissues, NF023 appeared to cause a ~10-fold rightward displacement of responses to NADPH, which would fit with its reported action as a competitive antagonist of P2X1 receptors.

DISCUSSION

Recently a number of studies have examined the effect of NADPH (and NADH) on vascular tone with a view to determining the role of NADPH oxidase in this response (Souza et al., 2001; Didion and Faraci, 2002; Paravicini et al., 2004; Park et al., 2004). However, here we have shown that contractile responses to NADPH in the mouse isolated aorta are not dependent on NADPH oxidase activity. Thus, although exogenous NADPH was highly effective at augmenting NADPH oxidase activity, its contractile effects were insensitive to diphenyleneiodonium and L-NAME, excluding any role for NADPH oxidase and superoxide-dependent inactivation of NO in the vasomotor response. Rather, vasocontractions to NADPH were mediated by activation of two distinct populations of P2X receptors, one located on the endothelium and the other on the smooth muscle layer.

In this study, as in previous studies by our group (Paravicini et al., 2002; Paravicini et al., 2004) and others (Pagano et al., 1995), exogenous NADPH stimulated superoxide production in intact vascular ring segments. Superoxide production was virtually abolished by diphenyleneiodonium and was markedly attenuated by apocynin suggesting that NADPH oxidase is primarily responsible for superoxide production in response to NADPH in mouse isolated aortic segments. These findings obtained using the lucigenin technique are thus consistent with those of Souza *et al.*, who, using electron paramagnetic spin resonance, similarly showed that extracellular NADPH stimulates superoxide production in intact aortic segments (Souza et al., 2001). Furthermore, these authors showed that superoxide production in response to NADPH was similar in aortas from wild-type and Nox2-knockout mice, implicating a non-phagocytic isoform of NADPH oxidase in the response (Souza et al., 2001). While it remains to be determined which isoform(s) is involved in supporting NADPH-dependent superoxide production

in whole ring segments, we have recently demonstrated a major role for a Nox4-containing NADPH oxidase in NADPH-dependent superoxide production in mouse cultured aortic smooth muscle cells (Ellmark et al., 2005). This is further supported by our observation that Nox4 is by far the most abundantly expressed NADPH oxidase isoform in the mouse aorta (Bengtsson et al., 2003).

In addition to elevating superoxide production, exogenous NADPH also caused concentration-dependent contractions in mouse aortic rings, which were partially dependent on the presence of a functional endothelium. However, our data indicates that NADPH did not mediate contraction via its ability to act as a reducing equivalent for NADPH oxidase-dependent superoxide generation and subsequent inactivation of NO. First, removal of the endothelium inhibited contractions to NADPH yet augmented superoxide production, this latter effect suggesting that endothelial cells exert a tonic suppressive effect on NADPH oxidase activity. Second, NADP^+ , which lacks a donatable hydride ion, was equally as effective as NADPH at causing contraction. Third, diphenyleneiodonium, which abolished NADPH oxidase-dependent superoxide production in mouse aortic rings, had no effect on NADPH-induced contractions. Finally, contractions to NADPH were not affected by the NOS inhibitor, L-NAME, indicating that they were independent of NO. Rather, both the endothelium-dependent and -independent components of the contractile response to NADPH were virtually abolished by the P2X receptor desensitising agent α,β -methylene-ATP and were markedly inhibited by the P2X antagonists, PPADS & NF023, implicating a role for P2X receptors in the response.

Although NADPH has previously been shown to display P2Y-like effects in the rat isolated mesenteric arterial bed (Ralevic and Burnstock, 1996), to our knowledge this is the first report that NADP(H) may act as a direct agonist at P2X receptors. P2X receptors are members of

the ligand-gated ion channel superfamily and are characterised by their inwardly rectified permeability to Ca^{2+} and other cations in response to ATP and related nucleotides. Within the vasculature, P2X1 appears to be the predominant P2X subtype expressed in smooth muscle cells (Kunapuli and Daniel, 1998; North, 2002). The P2X receptor profile of endothelial cells is less well characterised; however the predominant receptor subtypes on this cell type probably include P2X1 (Harrington and Mitchell, 2004), P2X4 (Glass et al., 2002), P2X5 (Schwiebert et al., 2002) and P2X7 (Ramirez and Kunze, 2002) receptors. Moreover, there is some evidence for regional variation (e.g. artery versus vein) in endothelial P2X receptor expression throughout the vasculature (Ray et al., 2002). Our finding that NF023, at a concentration which selectively targets P2X1 receptors (North, 2002), caused a ~3-fold rightward shift of the contraction response in endothelium-denuded rings certainly supports the idea that P2X1 are involved in the direct smooth muscle effects of NADPH. Responses to NADPH in rings with an intact endothelium were also partially impaired by NF023; however, given that we were unable to examine the endothelium-dependent component of the response in isolation from the direct smooth muscle effect of NADPH, it is not possible to conclude whether NF023 also has an effect at the endothelial level. Nevertheless we did find that PPADS and α,β -methylene-ATP virtually abolished both the endothelium-dependent and -independent components of the response to NADPH. α,β -methylene-ATP is selective for P2X1 and P2X3 receptor subtypes (North, 2002). Given that there is little evidence for P2X3 receptors on endothelial cells these findings with α,β -methylene-ATP probably indicate that the endothelial receptor involved is also of the P2X1 subtype.

While it is not surprising that activation of VSMC P2X receptors would lead to contractions via an increase in VSMC Ca^{2+} , it is somewhat surprising that stimulation of

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endothelial P2X receptors is also linked to contraction, since rises in endothelial cell Ca^{2+} are normally associated with release of endothelium-derived relaxing factors such as NO (and in certain vascular beds prostacyclin and endothelium-derived hyperpolarising factor). Previous studies have demonstrated that stimulation of endothelial P2Y receptors results in contraction via the release of thromboxane (Shirahase et al., 1991). However, the release of a vasoconstrictor prostanoid does not explain the endothelium-dependent vasocontraction effects to NADPH observed here as the cyclooxygenase inhibitor, indomethacin, had no effect (see Online Data Supplement - Figure 3). Hence, the mechanism by which P2X receptor stimulation leads to endothelium-dependent contraction remains to be elucidated.

In conclusion, here we have highlighted a novel mechanism(s) of endothelium-dependent and -independent vasocontraction to NADPH involving P2X receptors. As such, this study is the first to identify NADPH as a P2X receptor agonist. In addition, these findings should serve as a warning for future investigations that the effects of extracellular NADPH on vascular tone may not be solely attributable to NADPH oxidase activation.

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REFERENCES

- Bengtsson SH, Gulluyan LM, Dusting GJ and Drummond GR (2003) Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. *Clin Exp Pharmacol Physiol* **30**:849-854.
- Beny JL (2004) Characterization of purine receptors in mouse thoracic aorta. *J Cardiovasc Pharmacol* **44**:171-177.
- Burnstock G and Hoyle CH (1985) Actions of adenine dinucleotides in the guinea-pig taenia coli: NAD acts indirectly on P1-purinoceptors; NADP acts like a P2-purinoceptor agonist. *Br J Pharmacol* **84**:825-831.
- Burritt JB, DeLeo FR, McDonald CL, Prigge JR, Dinauer MC, Nakamura M, Nauseef WM and Jesaitis AJ (2001) Phage display epitope mapping of human neutrophil flavocytochrome b558. Identification of two juxtaposed extracellular domains. *J. Biol. Chem.* **276**:2053-2061.
- Didion SP and Faraci FM (2002) Effects of NADH and NADPH on superoxide levels and cerebral vascular tone. *Am J Physiol Heart Circ Physiol* **282**:H688-695.
- Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N and Drummond GR (2005) The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res* **65**:495-504.
- Glass R, Loesch A, Bodin P and Burnstock G (2002) P2X4 and P2X6 receptors associate with VE-cadherin in human endothelial cells. *Cell Mol Life Sci* **59**:870-881.
- Harrington LS and Mitchell JA (2004) Novel role for P2X receptor activation in endothelium-dependent vasodilation. *Br J Pharmacol* **143**:611-617.

- Kunapuli SP and Daniel JL (1998) P2 receptor subtypes in the cardiovascular system. *Biochem J* **336** (Pt 3):513-523.
- Lassegue B and Clempus RE (2003) Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* **285**:R277-297.
- Li JM and Shah AM (2001) Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. *Cardiovasc Res* **52**:477-486.
- Li JM and Shah AM (2002) Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* **277**:19952-19960.
- Madamanchi NR, Vendrov A and Runge MS (2005) Oxidative Stress and Vascular Disease. *Arterioscler Thromb Vasc Biol* **25**:29-38.
- Najbar A, Li CG and Rand MJ (1994) Pharmacological actions of the coenzymes NAD(H) and NADP(H) on the rat anococcygeus muscle. *Clin Exp Pharmacol Physiol* **21**:9-20.
- North RA (2002) Molecular Physiology of P2X Receptors. *Physiol. Rev.* **82**:1013-1067.
- Paclet MH, Henderson LM, Campion Y, Morel F and Dagher MC (2004) Localization of Nox2 N-terminus using polyclonal antipeptide antibodies. *Biochem J* **382**:981-986.
- Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI and Cohen RA (1995) An NADPH oxidase superoxide-generating system in the rabbit aorta. *Am J Physiol Heart Circ Physiol* **268**:H2274-2280.
- Paravicini TM, Chrissobolis S, Drummond GR and Sobey CG (2004) Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced cerebral vasodilatation to NADPH in vivo. *Stroke* **35**:584-589.

- Paravicini TM, Gulluyan LM, Dusting GJ and Drummond GR (2002) Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ Res* **91**:54-61.
- Park L, Anrather J, Zhou P, Frys K, Wang G and Iadecola C (2004) Exogenous NADPH increases cerebral blood flow through NADPH oxidase-dependent and -independent mechanisms. *Arterioscler Thromb Vasc Biol* **24**:1860-1865.
- Ralevic V and Burnstock G (1996) Discrimination by PPADS between endothelial P2Y- and P2U-purinoceptors in the rat isolated mesenteric arterial bed. *Br J Pharmacol* **118**:428-434.
- Ramirez AN and Kunze DL (2002) P2X purinergic receptor channel expression and function in bovine aortic endothelium. *Am J Physiol Heart Circ Physiol* **282**:H2106-2116.
- Ray FR, Huang W, Slater M and Barden JA (2002) Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selection of coronary artery grafts. *Atherosclerosis* **162**:55-61.
- Schwiebert LM, Rice WC, Kudlow BA, Taylor AL and Schwiebert EM (2002) Extracellular ATP signaling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells. *Am J Physiol Cell Physiol* **282**:C289-301.
- Shirahase H, Usui H, Shimaji H, Kurahashi K and Fujiwara M (1991) Endothelium-independent and endothelium-dependent contractions mediated by P2X- and P2Y-purinoceptors in canine basilar arteries. *J Pharmacol Exp Ther* **256**:683-688.
- Souza HP, Laurindo FR, Ziegelstein RC, Berlowitz CO and Zweier JL (2001) Vascular NAD(P)H oxidase is distinct from the phagocytic enzyme and modulates vascular reactivity control. *Am J Physiol Heart Circ Physiol* **280**:H658-H667.

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Effects of exogenous NADPH on superoxide production in mouse isolated aorta.

In (A) lucigenin-enhanced chemiluminescence was used to measure the concentration-dependent effect of NADPH on superoxide production in the absence and presence of the SOD inactivating agent DETCA (3mM). Lucigenin-enhanced chemiluminescence was also used to measure NADPH (100 μ M) -dependent superoxide production before and after treatment with (B) the superoxide scavenging compounds native SOD1 (300 U/mL) or tiron (10mM), and (C) following removal of the endothelium (E^-). All values are mean $\pm$ SEM of at least 4 experiments. * $P < 0.05$, ** $P < 0.01$ versus control.

Figure 2. Effect of exogenous NADPH on isometric tension in mouse isolated aorta. (A)

Original trace showing responses to cumulatively increasing half-log molar concentrations of NADPH in endothelium-intact rings of mouse aorta following precontraction to $\sim 50\%$ U_{max} with titrated concentrations of U46619. Acetylcholine (Ach; 30 μ M) was added at the end of the experiment to confirm the presence of a functional endothelium. Time bar represents 25 and 10 minutes before and after the break in the trace, respectively. (B) Group data showing responses to cumulatively increasing half-log molar concentrations of NADPH and (C) a single concentration of Ach (30 μ M) in endothelium-intact (control) and -denuded (E^-) tissues, and in the absence and presence of tiron. Values are mean $\pm$ SEM of at least 4 experiments. * $P < 0.05$, ** $P < 0.01$ versus control; $^{\Psi}P < 0.05$ versus endothelium-denuded; ns, not significant versus control.

Figure 3: Effect of NADPH, $NADP^+$ and NADH on superoxide production and isometric tension in mouse isolated aorta. In (A) lucigenin-enhanced chemiluminescence was used to measure superoxide production in the absence (control) or presence of NADPH (100 μ M), $NADP^+$ (100 μ M) or NADH (100 μ M). In (B) changes in isometric tension to cumulatively

increasing half-log molar concentrations of the three pyridine nucleotides following precontraction with U46619 are depicted. All values are mean $\pm$ SEM of at least 5 experiments. **P<0.01 versus control.

Figure 4. Effect of various oxidase inhibitors on NADPH-dependent superoxide production in mouse isolated aorta. Lucigenin-enhanced chemiluminescence was used to measure the effects of (A) acute and (B) chronic 24h incubation with the NADPH oxidase inhibitors diphenyleneiodonium (5 μ M) and apocynin (300 μ M). In (C) the effects of inhibitors of nitric oxide synthase (L-NAME; 100 μ M), xanthine oxidase (allopurinol; 100 μ M), cyclooxygenase (indomethacin; 3 μ M) and the mitochondrial electron transport chain (rotenone; 1 μ M) on NADPH-dependent superoxide production were examined. Data are mean $\pm$ SEM of at least 6 experiments. **P<0.01 versus control.

Figure 5. Effect of diphenyleneiodonium and L-NAME on changes in isometric tension responses to NADPH and Ach in mouse isolated aorta. Responses to (A) cumulatively increasing half-log molar concentrations of NADPH and (B) a single concentration of Ach (30 μ M) in U46619-precontracted mouse aortic rings in the absence (control) or presence of diphenyleneiodonium (5 μ M) or L-NAME (100 μ M). Values are mean $\pm$ SEM of at least 5 experiments. **P<0.01 versus control.

Figure 6. Effect of P2X receptor desensitisation on NADPH-dependent vasocontraction and superoxide production. The effect of α,β -methylene-ATP (10 μ M) was examined on (A) contractile responses to cumulatively increasing half-log molar concentrations of NADPH in both endothelium-intact and -denuded (E⁻) rings of aorta following precontraction with U46619, and (B) superoxide production in response to NADPH (100 μ M) in endothelium-intact tissues.

Values are mean $\pm$ SEM of at least 5 experiments. *P<0.05, **P<0.01 versus control; $^{\Psi}$ P<0.05 versus endothelium-denuded.

Figure 7. Effect of P2X antagonists on NADPH-dependent contractions in mouse isolated aorta. Effect of (A) PPADS (100 μ M) and (B) NF023 (10 μ M) on contractile responses to cumulatively increasing half-log molar concentrations of NADPH in both endothelium-intact and -denuded (E⁻) rings of aorta following precontraction with U46619. Values are mean $\pm$ SEM of at least 4 experiments. *P<0.05, **P<0.01 versus control. $^{\Psi}$ P<0.05 versus endothelium-denuded.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7