Chronic deficit in nitric oxide elicits oxidative stress and augments T-type calcium-channel contribution to vascular tone of rodent arteries and arterioles

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Received 24 July 2012; revised 11 February 2013; accepted 18 February 2013; online publish-ahead-of-print 22 February 2013

Time for primary review: 28 days

Aims
As cardiovascular disease is characterized by reduced nitric oxide bioavailability, our aim was to determine the impact of this change on the mechanism underlying vascular tone of pressurized arteries in vitro and in vivo.

Methods and results
We used pressurized cerebral and mesenteric arteries in vitro and skeletal muscle arterioles in vivo to study the contribution of L-type (1 μmol/L nifedipine) and T-type (1 μmol/L mibefradil, 3 μmol/L NNC 55–0396) calcium channels to vascular tone, following acute or chronic inhibition of nitric oxide. Acute inhibition with L-NAME (10 μmol/L) significantly increased the T-type, but not the L-type, channel contribution to vascular tone in vitro and in vivo, and altered the smooth muscle expression of the Ca v3.1 and Cav3.2 T-type channels. In pressurized mesenteric arteries of Cav3.1ko and Cav3.2ko mice, acutely treated with L-NAME, the contribution of T-type channels relative to L-type channels was significantly reduced, compared with arteries from wild-type mice. Chronic L-NAME treatment (40 mg/kg/day; 14–18 days) increased blood pressure, vascular superoxide, and the contribution of T-type channels to vascular tone in vivo. The latter was reversed by acute scavenging of superoxide with tempol (1 mmol/L), or inhibition of NADPH oxidase with apocynin (500 μmol/L) or DPI (5 μmol/L).

Conclusion
We conclude that nitric oxide deficit produces a significant increase in the contribution of Ca v3.1 and Ca v3.2 T-type calcium channels to vascular tone, by regulating the bioavailability of reactive oxygen species produced by NADPH oxidase. Our data provide evidence for a novel causal link between nitric oxide deficit, oxidative stress, and T-type calcium channel function.

Keywords
T-type calcium channels • Nitric oxide • Reactive oxygen species • Vascular tone

1. Introduction
Vasoconstriction in response to increased intraluminal pressure and sympathetic nerve activation is initiated by depolarization of vascular smooth muscle cells and calcium entry through voltage-dependent calcium channels.1–4 Of the 10 molecular subtypes, the L-type channels are known to supply the calcium necessary for persistent vascular tone in both cerebral and systemic circulations5–8 and enhanced activity of L-type channels is linked to hypertension and cerebrovascular disease.9 However, the T-type channels, Ca v3.1 and Ca v3.2, have recently been shown to be expressed in the renal, mesenteric, cerebral, skeletal muscle, and subcutaneous circulations and pharmacological studies have suggested that they contribute in combination with L-type channels to vascular tone of small arteries and arterioles.8–16

Endothelial dysfunction, a hallmark of hypertension and cardiovascular disease, is characterized by reduced nitric oxide bioavailability and increased oxidative stress, the latter resulting from up-regulated activity of a family of enzyme complexes called NADPH oxidases.17,18 Indeed, oxidative stress is generally considered to be responsible for the reduction in nitric oxide bioavailability, due to superoxide scavenging of nitric oxide as well as uncoupling of endothelial nitric oxide synthase, following reduction in its co-factor tetrahydrobiopterin.19,20

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In wire myograph-mounted segments of middle cerebral arteries, acute inhibition of nitric oxide synthesis with N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), has been shown to elicit vasodistension which was partially blocked by T-type channel antagonists, however, the relevance of these data to the control of cerebrovascular tone under pressurized conditions, or to the control of peripheral vascular tone under physiological or pathophysiological conditions is unknown.

Our aim was three-fold: first, to determine the effect of acute inhibition of nitric oxide on the contribution of L- and T-type channels to vascular tone of pressurized cerebral arteries in vitro and systemic arterioles in vivo; secondly, to use Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 knockout mice to attribute effects of pharmacological blockade to molecular T-type channel subtypes, and thirdly, to investigate the effects of chronic nitric oxide inhibition, as occurs during cardiovascular disease, through the use of the L-NAME-treated animal model of hypertension.

## 2. Methods

Experiments were performed on Wistar rats (6–8 weeks) and C57BL/6 wild-type mice, Ca\textsubscript{v}3.1 knockout (Ca\textsubscript{v}3.1ko), and Ca\textsubscript{v}3.2 knockout mice (Ca\textsubscript{v}3.2ko) 8–12 weeks, according to protocols approved by the Australian National University Animal Experimentation Ethics Committee and the Danish Animal Experiments Inspectorate of the Danish Ministry of Justice, under the guidelines of the National Health and Medical Research Council of Australia (NHMRC) and National Institutes of Health (NIH Publication, 8th Edition, 2011), respectively. Male and female Ca\textsubscript{v}3.2ko and their wild-type littermates were obtained from heterozygous breeding (Mutant Mouse Regional Resource Centers, MMRRC, Columbia, MO, USA). In studies with Ca\textsubscript{v}3.1ko, C57BL/6 wild-type mice were used as controls (Taconic Farm Inc., Ry, Denmark) since Ca\textsubscript{v}3.1ko mice were back-crossed to a C57BL/6 background for more than 10 generations.

In some experiments, Ca\textsubscript{v}3.1ko and Ca\textsubscript{v}3.2ko mice were treated for 14–18 days with L-NAME (0.5 mg/mL, equivalent to 40 mg/kg/day for a 25 g mouse drinking 2 mL/day; Sigma-Aldrich, St Louis, MO, USA) in the drinking water. Blood pressure and heart rate were measured using tail cuff plethysmography on days 6, 8, and 9 of L-NAME treatment.

### 2.1 Cremaster muscle arteriolar preparation

Mice were anesthetized intraperitoneally (1 mg/kg medetomidine, 10 mg/kg midazolam; Pfizer Australia Pty Ltd, NSW, Australia, 0.1 mg/kg fentanyl; Mayne Pharma Limited, Victoria, Australia) and continuously infused via a jugular vein catheter (medetomidine 0.02 mg/h; midazolam 0.2 mg/h; fentanyl 0.002 mg/h). Adequacy of anesthesia was monitored by pedal response, according to animal Ethics Guidelines. Body temperature was maintained at ~37°C by a heating pad and mice were intubated with a polyethylene cannula to facilitate breathing. The right cremaster muscle was carefully dissected and spread over a coverslip to enable visualization of arterioles and superfused (3 mL/min) with Krebs solution (mmol/L: Na\textsubscript{Cl} 3.8 KCl 25 NaH\textsubscript{2}CO\textsubscript{3} 1.2 KH\textsubscript{2}PO\textsubscript{4} 2.5 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, gassed with 5% CO\textsubscript{2}/95%N\textsubscript{2}, pH 7.4, at 34°C.

L-NAME (10 μmol/L) in the superfusate for 30 min was used to acutely inhibit physiologically active nitric oxide, as preliminary data showed that subsequent addition of the nitric oxide scavenger, hydroxocobalamin (100 μmol/L), had no further effect on vascular tone (Total tone: L-NAME: 60 ± 6.6 100-%/D\textsubscript{max}, L-NAME + hydroxocobalamin: 60 ± 6.5 100-%/D\textsubscript{max}, n = 31 arterioles/three mice). Our previous studies have shown that L-NAME treatment has no effect on resting membrane potential of either smooth muscle or endothelial cells of cremaster arterioles. The contribution of L- and T-type channels to vascular tone was determined in the absence and the presence of L-NAME using nifedipine...

## 2.2 Pressure myography

Primary side branches of the basilar artery were removed from rats anesthetized with 4% isoflurane in oxygen and decapitated. Arterial segments were cannulated, pressurized, and equilibrated in L-NAME or the control inactive isomer, D-NAME (10 μmol/L). Pressure–response curves were determined from 10 to 120 mmHg and repeated in nifedipine (1 μmol/L, Sigma-Aldrich, St Louis, MO, USA), then nifedipine and mibebradil (1 μmol/L, 14,33). Mibebradil was always added after nifedipine to prevent effects of non-specific action on L-type channels. 14,34 The role of L- and T-type channels was normalized to L- and T-type channels which was determined in calcium-free solution containing EGTA (1 mmol/L).

Second-order mesenteric arteries were dissected from mice killed by cervical dislocation. Arterial segments were cannulated and pressurized to 60 mmHg with PSS containing 1% bovine serum albumen. After equilibration for 30 min, vessels were superfused with L-NAME (10 μmol/L) for 1 h. Since these vessels did not exhibit spontaneous vascular tone, vessels were pre-constructed with phenylephrine (1 μmol/L) to simulate neuro-vascular tone, and then incubated cumulatively in nifedipine (1 μmol/L) and NNC 55–0396 (30 min) of reactive oxygen species (ROS) with the NADPH oxidase inhibitors 4′-Hydroxy-3′-methoxyacetophenone (apocynin, 500 μmol/L, Sigma-Aldrich, St Louis, MO, USA) or diphenyleeniodion (DPI, 5 μmol/L, 33,32 Sigma-Aldrich, St Louis, MO, USA), or the superoxide dismutase mimetic, 4-Hydroxy-TEMPOL (tempol, 1 mmol/L, Sigma-Aldrich, St Louis, MO, USA). The role of exogenous ROS was tested with hypoxanthine (250 μmol/L) and xanthine oxidase (3 μmol/L). Arteries were superfused with hypoxanthine, and then with hypoxanthine and xanthine oxidase together. Maximal vessel diameter (D\textsubscript{max}) was determined at the end of the experiments by superfusion of Krebs solution containing SNP, acetylcholine (ACh, Sigma-Aldrich, St Louis, MO, USA), and adenosine (Merck, Kilsyth, Victoria, Australia; 30 μmol/L each). Mice were euthanized by cervical dislocation without regaining consciousness.

Vascular tone was calculated as 100 × (D\textsubscript{max} - D\textsubscript{0})/D\textsubscript{max} where D\textsubscript{0} represents resting diameter. The contribution of L- and T-type channels to vascular tone was normalized to D\textsubscript{max} in order to account for variability in arteriolar diameter of individual arterioles. The contribution of L-type channels was determined as [100 × (D\textsubscript{nifedipine} - D\textsubscript{0})/D\textsubscript{max}] while the percentage contribution of T-type channels was determined by [100 × (D\textsubscript{nifedipine} + NNC - D\textsubscript{nifedipine})/D\textsubscript{max}]. In order to determine the average contribution of L- and T-type channels to vascular tone across all arterioles of the cremaster muscle microcirculation, the percentage contribution of L- and T-type channels was normalized to vasodilator mediator (D\textsubscript{max} - D\textsubscript{0})

## 2.3 Immunohistochemistry

Basilar arteries were incubated in L-NAME (10 μmol/L) in PSS for 30 min at 37°C and smooth muscle cells isolated, fixed in 2% paraformaldehyde, and stained with antibodies against Ca\textsubscript{v}3.1 (1:500; kindly provided by Dr L.L.Cribbs, Loyola University, Chicago, IL, USA). Staining was quantified in defined areas in the cytoplasm and near the membrane, using the thresholding function of ImageJ.

Intact cremaster muscles were isolated from mice euthanized by cervical dislocation, pinned flat, fixed, stained with rabbit anti-Ca\textsubscript{v}1.2...
(1:300), anti-Ca3.1 (1:500), or anti-Ca3.2 (1:300, kindly provided by Dr LL.Cribbs) and analysed with ImageJ.

2.4 Dihydroethidium staining

Control mice and mice chronically treated with l-NAME were anaesthetized with isoflurane, decapitated and cremaster arterioles and surrounding skeletal muscle, as well as mesenteric arteries removed. Cryostat sections were incubated with or without dihydroethidium (2 μmol/L, Sigma-Aldrich, St Louis, MO, USA) and nuclear staining intensity analysed with ImageJ. In order to determine whether superoxide was the main product involved in the fluorescent signal, additional sections were incubated in dihydroethidium, with or without polyethylene glycol-superoxide dismutase (PEG-SOD; 250 Units/mL).

Detailed Methods are provided in Supplementary material online.

3. Results

3.1 Acute inhibition of nitric oxide increases the contribution of T-type channels to tone of pressurized cerebral arteries in vitro

Acute treatment of pressurized basilar arterial branches ($D_{\text{max}} = 189 \pm 6 \, \mu m$, $n = 10$) with L-NAME (10 μmol/L) produced a significant leftward shift in the pressure–response curve (Figure 1A) and significant increase in the contribution of T-type channels to vascular tone at all intraluminal pressures, compared with treatment with the inactive control, d-NAME (Figure 1C). This was accompanied by a significant decrease in the contribution of L-type channels from 40 to 120 mmHg (Figure 1B).

3.2 Acute inhibition of nitric oxide increases membrane expression of Ca3.1 in isolated cerebrovascular smooth muscle cells

Incubation of cerebral vessels in l-NAME at 37°C led to a significant redistribution of Ca3.1 staining from the cytoplasm in the absence of l-NAME, to a region near the cell membrane (Figure 2A and B). Incubation of vessels in l-NAME at room temperature failed to significantly alter distribution (Figure 2A and B) suggesting an energy-dependent mechanism. No staining was seen without the primary antibody. Experiments were not conducted with antibodies against Ca3.2 due to a failure to produce significant staining of isolated smooth muscle cells.14

3.3 Acute inhibition of nitric oxide increases the contribution of T-type channels to tone of cremaster muscle arterioles in vivo

Cremaster arterioles were divided into four groups according to the degree of vascular tone (Figure 3A–D): the smallest arterioles having the greatest tone while the largest arterioles having the least tone (70–100% tone, $D_{\text{max}} = 27.6 \pm 3.9 \, \mu m$, 16 arterioles/seven mice; 50–69% tone, $D_{\text{max}} = 35.5 \pm 4.4 \, \mu m$, 32 arterioles/nine mice; 30–49% tone, $D_{\text{max}} = 44.2 \pm 4.0 \, \mu m$, 29 arterioles/nine mice; 1–29% tone, $D_{\text{max}} = 67.3 \pm 5.8 \, \mu m$, 30 arterioles/eight mice). L-type channels contributed substantially to tone in all groups, while the contribution of T-type channels decreased with increasing vessel size (Figure 3A–D).

Acute superfusion with l-NAME (10 μmol/L) had no effect on tone of the two smallest arterial groups with the highest tone (Figure 3A and B), but significantly increased tone in the two larger groups (Figure 3C and D). While the contribution of L-type channels was not affected by acute l-NAME treatment, the contribution of T-type channels was significantly increased in all but the smallest arteriolar group (Figure 3B–D, cf. 3A).

In order to combine data from all arterioles in spite of varying degrees of vascular tone, we normalized expression of L- and T-type channels in each arteriole to vascular tone. Combined data...
showed that acute L-NAME treatment significantly increased mean vessel tone and the relative contribution of T-type channels, but reduced the contribution of L-type channels ($P < 0.05$; Figure 3E and F).

The effect of acute L-NAME treatment on the contribution of L-type and T-type channels was reversed by superfusion with the nitric oxide donor, SNP (100 nmol/L; Supplementary material online, Figure S1).

### 3.4 Acute inhibition of nitric oxide increases expression of T-type channels in cremaster muscle arterioles

Immunohistochemical staining for $\text{Ca}v_{1.2}$, L-type channels was abundant in smooth muscle cells of cremaster arterioles, with no significant change following acute incubation with L-NAME (Figure 4A and B; Supplementary material online, Figure S2; $P > 0.05$). In contrast, $\text{Ca}v_{3.1}$ staining was weak in smooth muscle cells under control conditions, but significantly increased after incubation with L-NAME (Figure 4C and D; Supplementary material online, Figure S2; $P < 0.05$). Staining for $\text{Ca}v_{3.2}$ was frequently absent from smooth muscle cells of cremaster arterioles, but became apparent after L-NAME exposure (Figure 4E and F; Supplementary material online, Figure S2; $P < 0.05$). No staining was observed without the primary antibody.

### 3.5 Contribution of T-type channels to vascular tone is less in arteries from $\text{Ca}v_{3.2}^{\text{ko}}$ mice than in wild-type mice following acute L-NAME treatment

L- and T-type channels contributed equally to phenylephrine (1 μmol/L)-induced vascular tone of second-order mesenteric...
arteries from wild-type mice under conditions of acute inhibition of nitric oxide (Figure 5A). In contrast, in arteries isolated from Cav3.1ko and Cav3.2ko mice, the contribution to tone of T-type channels was significantly smaller than that of L-type channels (P < 0.05; Figure 5B and C) and for Cav3.2ko arteries, the contribution of T-type channels was significantly less than that in wild-type vessels (P < 0.05; Figure 5C, cf. 5A). Although there was a trend to an increase in the contribution of L-type channels to tone in arteries from both Cav3.1ko and Cav3.2ko mice, this was not statistically significant (P > 0.05).

3.6 Chronic inhibition of nitric oxide increases the contribution of T-type channels to tone of cremaster muscle arterioles in vivo

Chronic exposure of wild-type mice to l-NAME for 14–18 days caused a significant rise in blood pressure which was maximal by

Figure 4 Expression of T-type channels in mouse cremaster arterioles is increased by acute l-NAME treatment. (A and B) L-type channel expression (green) in smooth muscle cells (SMCs) of arterioles of untreated mice (NT; no treatment) is unaltered by acute l-NAME treatment (l-NAME). Nuclei of SMCs (red) lie perpendicular to the longitudinal vessel axis (dotted lines). (C–F) Expression of T-type channels (green), CaV3.1 (C and D), and CaV3.2 (E and F) is increased in SMCs by acute l-NAME treatment (D and F, cf. C and E). Nuclei of endothelial cells (ECs) lie parallel with the vessel axis.

Figure 5 Contribution of T-type channels to vascular tone is less in arteries from Cav3.2ko than wild-type mice following acute l-NAME treatment. (A) L- and T-type channels contribute equally to phenylephrine-induced vascular tone of mesenteric arteries from wild-type mice. (B and C) In mesenteric arteries from Cav3.1ko and Cav3.2ko mice, the contribution of T-type channels was significantly less than that of L-type channels. T-type channel involvement in Cav3.2ko arteries was also significantly less than that in wild-type arteries. Data represent means ± SEM for six arteries (wild-type), six arteries (Cav3.1ko), eight arteries (Cav3.2ko). *P < 0.05 significant difference between L- and T-type contributions. **P < 0.05 significantly different from corresponding wild-type value.
Day 6 of treatment (Day 6: L-NAME: 121 ± 5 mmHg; Day 9: L-NAME: 121 ± 3 mmHg, n = 5 mice; P > 0.05; cf. no treatment: 107 ± 5 mmHg; P < 0.05). Heart rate did not vary between L-NAME-treated and untreated mice (L-NAME: 603 ± 30 bpm, n = 5 mice; untreated: 670 ± 30 bpm, n = 5 mice; P > 0.05).

When data for all vessels were considered, the relative contribution of T-type channels to tone was significantly increased by chronic L-NAME treatment, compared with untreated mice, without significant change in either L-type channels or vascular tone (Chronic L-NAME: Supplementary material online, Figure S3A–D, cf. NT: Figure 3A–D). In contrast, the contribution of L-type channels to tone was unchanged in the three smaller vessel groups (P > 0.05) but significantly decreased in the largest vessel group (Chronic L-NAME: Supplementary material online, Figure S3A–D, cf. NT: Figure 3A–D; P < 0.05).

### 3.7 Oxidative stress is causal to the increased contribution of T-type channels following chronic inhibition of nitric oxide

Acute treatment with the superoxide dismutase mimetic, tempol (1 mmol/L), of cremaster arterioles of mice chronically treated with L-NAME, significantly reduced tone (Figure 6A) and reversed the increased contribution of T-type channels to vascular tone (Figure 6B). Analysis of vessel sub-groups showed that acute tempol treatment significantly reduced tone in all, but the largest vessel group with the least tone (Supplementary material online, Figure S3A–D). The contribution of T-type channels to tone was significantly reduced in all groups, while the contribution of L-type channels was significantly reduced in the smallest vessel group but not in the three other groups (Supplementary material online, Figure S3A–D). Control experiments in cremaster arterioles of mice acutely superfused with tempol, but not subjected to chronic treatment with L-NAME, showed that both vascular tone and the contribution of L- or T-type channels were not significantly different from untreated mice (Vessel tone: 41.2 ± 7.3 100-%D/Dmax; L-type, 63.9 ± 5.9% contribution to vessel tone; T-type, 23.7 ± 3.4% contribution to vessel tone; 42 arterioles/four mice; P > 0.05 cf. NT; Figure 3E and F).

In order to test a role for NADPH oxidases in the changes in calcium-channel subtypes initiated by chronic L-NAME treatment, we conducted further experiments using the NADPH oxidase inhibitor, apocynin, and the flavin inhibitor, DPI, acutely on cremaster arterioles. Consistent with the data obtained with tempol, both drugs caused a significant reduction in vascular tone and the relative contribution of T-type channels (Figure 6C and D).

When cremaster muscle arterioles were analysed according to the degree of vascular tone (Supplementary material online, Figure S4A–D: 70–100% tone, Dmax = 27.1 ± 1.6 μm, 20 arterioles/10 mice; 50–69% tone, Dmax = 40.8 ± 2.2 μm, 51 arterioles/14 mice; 30–49% tone, Dmax = 49.7 ± 2.6 μm, 52 arterioles/13 mice; 1–29% tone, Dmax = 82.7 ± 5.1 μm, 32 arterioles/10 mice), both drugs significantly reduced vascular tone and the T-type channel involvement in all but the largest vessel group (Supplementary material online, Figure S4A–D).

The role of ROS in altering the contribution of T-type channels was confirmed by superfusion of untreated arterioles with the ROS generating system, hypoxanthine and xanthine oxidase. Exogenous application of hypoxanthine led to a decrease in vessel tone without change in the relative contribution of L- and T-type channels (Supplementary material online, Figure S5). Addition of xanthine oxidase to hypoxanthine did not affect vascular tone, but significantly reduced the contribution of L-type channels and significantly increased the contribution of T-type channels (Supplementary material online, Figure S5). Addition of hypoxanthine and xanthine oxidase, in the presence of L-NAME, did not significantly increase the T-type channel contribution to vascular tone, compared with that already elicited by elimination of nitric oxide (i.e. L-NAME: Vessel tone: 56.5 ± 2.7 100-%D/Dmax; L-type, 44.7 ± 9.0% contribution to vessel tone; T-type, 34.9 ± 3.0% contribution to vessel tone; L-NAME +...
hypoxanthine + xanthine oxidase: Vessel tone: 43.0 ± 2.7 100-%D/D0max: L-type, 45.2 ± 8.7% contribution to vessel tone; T-type, 50.3 ± 9.0% contribution to vessel tone; 41 arterioles/four mice).

3.8 Chronic inhibition of nitric oxide increases superoxide in vascular smooth muscle cells

Chronic exposure to L-NAME significantly increased dihydroethidium staining of smooth muscle cell nuclei in both cremaster arterioles and mesenteric arteries, compared with those of untreated wild-type mice (Supplementary material online, Figure S6; cremaster arterioles: Chronic L-NAME: 60 ± 4 pixel intensity, n = 49 nuclei/five arteries; NT: 35 ± 7 pixel intensity, n = 43 nuclei/five arteries; P < 0.05; mesenteric arteries: Chronic L-NAME: 56 ± 8 pixel intensity, n = 84 nuclei/12 arteries; NT: 30 ± 5 pixel intensity, n = 72 nuclei/11 arteries; P < 0.05). Control sections incubated without dihydroethidium showed no nuclear labelling.

Incubation of arterial sections with dihydroethidium in the presence of PEG-SOD significantly reduced the staining intensity in both vessels, indicating that superoxide was the main fluorescent product (cremaster arterioles: Chronic L-NAME + PEG-SOD: 23 ± 4 pixel intensity, n = 25 nuclei/four arteries; chronic L-NAME – PEG-SOD: 64 ± 12 pixel intensity, n = 48 nuclei/five arteries; P < 0.05; mesenteric arteries: Chronic L-NAME + PEG-SOD: 37 ± 3 pixel intensity, n = 58 nuclei/six arteries; chronic L-NAME – PEG-SOD: 84 ± 8 pixel intensity, n = 49 nuclei/six arteries; P < 0.05).

4. Discussion

The present study provides a novel insight into how nitric oxide modulates peripheral vascular tone. The major findings are (i) that acute and chronic inhibition of nitric oxide produces a significant increase in T-type channel involvement in resistance vessels, (ii) that this change in electromechanical coupling results from increased bioavailability of superoxide produced from NADPH oxidase, and (iii) that the effects result from specific actions on the molecular T-channel subtypes, Ca3.1 and Ca3.2. Together these data provide the first evidence that nitric oxide plays an active role in limiting the bioavailability of ROS. Under pathophysiological conditions of reduced nitric oxide bioavailability, superoxide levels are increased and the T-type calcium-channel contribution to peripheral vascular tone is augmented, due to increased expression and/or redistribution of channels to the cell membrane.

4.1 Acute nitric oxide deficit increases vascular tone through recruitment of T-type channels

Our initial studies investigated the effects of acute inhibition of nitric oxide on the contribution of L- and T-type channels to vascular tone of pressurized vessels. We found that vascular tone was increased in cerebral arteries in vitro and in skeletal muscle arterioles in vivo, and that this resulted from a selective increase in the contribution of T-type channels, but not L-type channels (Figures 1 and 3). The full reversal of this effect following superfusion with SNP (Supplementary material online, Figure S1) suggests a negative regulation of T-type channel function by nitric oxide. In the cerebral vessels, the increased involvement of T-type channels was accompanied by a significant decrease in the contribution of L-type channels at intraluminal pressures >40 mmHg. Our data obtained from cerebral myocytes showed that the rapidity of this change in electromechanical coupling could be explained by increased trafficking of T-type channels from the cytoplasm to the cell membrane (Figure 2), while in skeletal muscle arterioles in situ, acute nitric oxide inhibition significantly increased protein expression of T-type channels in smooth muscle cells (Figure 4; Supplementary material online, Figure S2). The increased activity of Ca3.2 channels in nociceptive primary sensory neurones in a model of irritable bowel syndrome has also been attributed to rapid insertion of channels into the cell membrane.35

In the cremaster muscle microcirculation in vivo, the change in electromechanical coupling following nitric oxide deficit was found in all except the smallest arterioles, where nitric oxide did not play any role in regulating vascular tone and T-channels contributed to vasoconstriction under physiological conditions (Figure 3A). Such a contribution of T-type channels to normal vascular tone has been found in small vessels of other vascular beds.14,16,36 Coupled with a diminishing role for nitric oxide in endothelial vasoilation as vessel size decreases,37,38 these data support the proposal that nitric oxide negatively regulates T-type channel function.
may release this suppression of NADPH-oxidase, thereby increasing superoxide synthesis. However, a comprehensive study of the role of potential downstream signalling molecules activated by nitric oxide and their effects on the regulation of bioactive ROS is needed to validate these predictions. The role of ROS in controlling T-type channel function was confirmed by the increased T-type channel contribution to vascular tone induced by exogenous ROS production from xanthine oxidase (Supplementary material online, Figure S5). The absence of a significant increase in the T-type channel contribution induced by exogenous ROS over those effects already induced by inhibition of nitric oxide with L-NAME, suggests that the effects of ROS in increasing T-channel involvement were already maximal.

Recent studies in chromaffin cells, cardiomyocytes, and sensory neurones have provided evidence for redox regulation of both T-type channel mRNA expression and T-type channel activity. Although arterial L-type channel activity has also been reported to be up-regulated by ROS, we did not find any such effects on L-type channels here. However, our data indicate that superoxide up-regulates T-type channel activity, in contrast to the hydrogen peroxide-mediated stimulation of arterial L-type channels. The recent demonstration of hydrogen peroxide-mediated inhibition of T-type channel activity suggests a complex regulation of calcium-channel activity by different ROS.

While the effects described here are indicative of a role for NADPH oxidase activity, it should be noted that apocynin can have direct antioxidant actions and DPI will inhibit the activity of other flavin containing enzymes. Future studies in mice deficient in specific NADPH oxidase subtypes will therefore be necessary to delineate the involvement of these different isoforms in the increased superoxide production. Nevertheless, the present study has demonstrated nitric oxide deficit as a direct or indirect cause of increased oxidative stress; a feedback loop which could exacerbate tissue damage.

4.3 Ca\textsubscript{3.1} and Ca\textsubscript{3.2} are the molecular targets for T-type channel antagonism

T-type channels have been attributed a role in vasoconstriction of small vessels in a number of vascular beds, due to arterial expression of the molecular channel subtypes, Ca\textsubscript{3.1} and Ca\textsubscript{3.2}, and reduction in vasomotor responses following application of T-type channel antagonists. However, many of the T-type pharmacological agents used previously have been reported to lack specificity and therefore a role for T-type channels in vascular tone remains controversial. We reasoned that if the pharmacological antagonist, NNC 55–0396, was selective for the molecular T-channel subtypes, then it should have a reduced effect on the residual vascular tone of arteries from Ca\textsubscript{3.1}ko, or Ca\textsubscript{3.2}ko mice, after blockade of L-type channels with nifedipine. Our data obtained in mesenteric arteries of Ca\textsubscript{3.1}ko or Ca\textsubscript{3.2}ko mice confirm these predictions, demonstrating for the first time the involvement of Ca\textsubscript{3.2} channels, and to a lesser degree, Ca\textsubscript{3.1} channels, in vascular tone during nitric oxide deficiency (Figure 5).

4.4 Summary

We show that under normal physiological conditions in vivo, T-type channels contribute to vascular tone of the smallest arterioles, in which nitric oxide does not play a role in vasodilation. In contrast, in larger vessels, chronic inhibition of nitric oxide increases T-type channel contribution to peripheral vascular tone, due to augmentation of superoxide bioavailability through the NADPH oxidase pathway. We further show that the effects of the T-type channel antagonist, NNC 55–0396, are mediated by the Ca\textsubscript{3.1} and Ca\textsubscript{3.2} T-type molecular subtypes and that the increased contribution of these channels results from increased expression or redistribution of protein to the cell membrane. Interestingly, the central region of the I–II loop of both Ca\textsubscript{3.1} and Ca\textsubscript{3.2} has been identified as integral to the regulation of surface expression of these channels and may be the target for these effects. Importantly, we provide evidence for a novel causal link between nitric oxide deficit, increased oxidative stress, and T-type channel function. Future studies should be directed to identifying the NADPH oxidase subtypes involved in these effects and the mechanism(s) by which superoxide augments T-type channel activity.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Dr Stephanie Wölfe for contributing immunohistochimical data for Figure 4, Mr Kenneth Andersen for technical assistance with data in Figure 5, and Dr Philippe Lory for back-crossing the Ca\textsubscript{3.1}ko into the C57BL/6 background.

Conflict of interest: none declared.

Funding

This work was supported by the National Health and Medical Research Council of Australia (471420), Heart Foundation (G12C 6361) and the Danish Medical Research Council (11–107552).

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