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RESEARCH ARTICLE

NADPH oxidase derived ROS promote arterial contraction in early postnatal rats by activation of L-type voltage-gated Ca^{2+} channels

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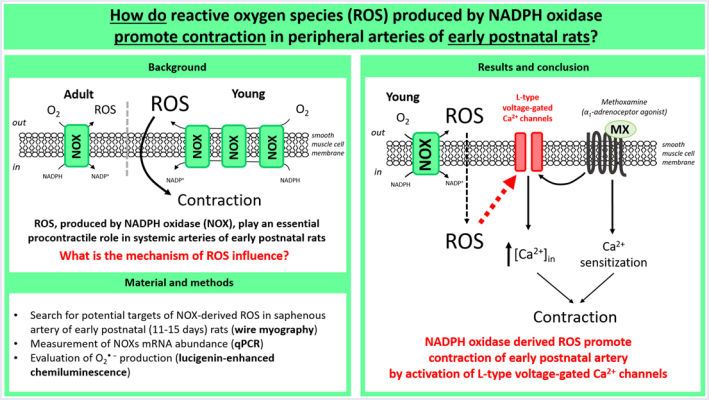
ABSTRACT

Reactive oxygen species (ROS) produced by NADPH oxidase promote contraction of peripheral arteries, which is especially pronounced in early postnatal period in comparison to adulthood, but the mechanisms of such vasomotor influence are poorly understood. We tested the hypothesis that Rho-kinase and protein kinase C (PKC) mediate procontractile influence of NADPH oxidase derived ROS in peripheral artery of early postnatal rats. In addition, we evaluated the involvement Src-kinase and L-type voltage-gated Ca^{2+} channels (LTCC) into procontractile influence of ROS, produced by NADPH oxidase, because of their known interplay with Rho-kinase and PKC pathways. Saphenous arteries from 11- to 15-day-old male rats were studied using quantitative PCR, isometric myography and lucigenin-enhanced chemiluminescence. Arterial tissue of early postnatal rats contained *Nox2*, *Nox4*, *Duox1* and *Duox2* mRNAs, among which *Nox2* mRNA was the most abundant. Pan-NADPH oxidase inhibitor VAS2870 (10 µM) significantly reduced arterial contractile responses to methoxamine. The inhibitors of Rho-kinase (Y27632, 3 µM), PKC (GF109203X, 10 µM) and Src-kinase (PP2, 10 µM), as well as LTCC blockers (nimodipine, 0.1 µM, and verapamil, 0.1 µM) also reduced methoxamine-induced contraction. Importantly, the effect of VAS2870 persisted in the presence of Rho-kinase, PKC or Src-kinase inhibitors, but not in the presence of LTCC blocker. Notably, the blockade of LTCC did not affect either basal or NADPH-induced $O_2^{\bullet-}$ production. Our data show that LTCC, but not Rho-kinase, PKC or Src-kinase are involved into procontractile effect of ROS, produced by NADPH oxidase, in saphenous artery of young rats. Calcium influx through LTCC does not activate ROS production by NADPH oxidase.

HIGHLIGHTS

- NOX-derived ROS have a strong procontractile influence in rat pup arteries
- NOX-derived ROS contract pup arteries regardless of Rho-kinase, PKC and Src-kinase
- NOX-derived ROS contract pup arteries by activation of L-type Ca^{2+} channels

GRAPHICAL ABSTRACT



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NADPH oxidase; Rho-kinase; L-type voltage-gated Ca^{2+} channels; artery; early postnatal ontogenesis

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1. Introduction

During last years, increasing evidence has been accumulating that reactive oxygen species (ROS) play an important role in normal regulation of vascular tone [1–3]. NADPH oxidase make a significant contribution to ROS production in the vasculature [1,2].

The mechanisms of ROS influence on vascular smooth muscle tone are not fully understood and continue to be the subject of intensive research. The mechanisms of ROS procontractile effect known to date include the activation of intracellular signaling pathways involving Rho-kinase [4,5], Src-kinase [6], mitogen-activated protein kinase (MAPK) [7,8] or protein kinase C (PKC) [9]. Of note, the same enzyme, for example PKC or Src-kinase, can both activate NADPH oxidase and mediate the procontractile effects of NADPH oxidase produced ROS [2]. Besides that, ROS were shown to augment contraction by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) upon activation of L-type voltage-gated Ca^{2+} channels (LTCC) [10,11] or transient receptor potential channels, type C (TRPC) [12], as well as through deactivation of voltage-gated potassium channels (Kv) [13].

Notably, all the mechanisms discussed above were described for an adult mature organism. However, our recent studies have shown that ROS produced by NADPH oxidase play an essential vasomotor role in systemic arteries of 1–2-week-old rats, while their influence was not observed in the same type of arteries in adult rats [14]. Nevertheless, the mechanisms of the vasomotor influence of ROS produced by NADPH oxidase in systemic arteries during the early postnatal period remain unclear.

It should be noted that many mechanisms regulating vascular contraction in the early postnatal period differ significantly from that in adulthood. In general, the contribution of Ca^{2+}-dependent mechanisms to the regulation of contraction is smaller, while the contribution of Ca^{2+}-sensitization is higher in arteries of 1–2-week-old rats [15]. The established players in the development of Ca^{2+}-sensitization are Rho-kinase and PKC [16]. Obviously, Rho-kinase contribution to arterial contraction is considerably higher in early postnatal ontogenesis compared to the adulthood [17–19]. The contribution of PKC can be increased as well, but not as significantly as Rho-kinase, and depending on the way of arterial contraction induction [18,19]. At the same time, MAPK do not contribute to the regulation of systemic artery contraction in early postnatal ontogenesis [20].

Considering the pronounced procontractile influence of Rho-kinase and, to some extent, PKC in developing rat pup arteries and known involvement of these kinases in vasomotor effects of ROS, we hypothesized that Rho-kinase and PKC are substantially involved in procontractile action of ROS produced by NADPH oxidase in systemic arteries during early postnatal ontogenesis. To test this hypothesis, we studied arterial contractile responses of 11-15-day-old rat pups in the presence of pan-NADPH oxidase inhibitor VAS2870 in combination with Rho-kinase inhibitor Y27632 or PKC inhibitor GF109203X. In addition, we evaluated the involvement Src-kinase and LTCC into procontractile influence of ROS produced by NADPH oxidase, because of known interplay of these mechanisms with Rho-kinase and PKC pathways [21,22].

2. Methods

2.1. Animals

Animal studies were performed in compliance with International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences. The use of laboratory animals and all procedures used in this study was approved by the Biomedical Ethics Committee of M.V. Lomonosov Moscow State University (Protocol number 149-g). Wistar rats (obtained from the Institute of General Pathology and Pathophysiology, Russia) were used in this study. Rats were maintained with controlled temperature and a 12/12 h light/dark cycle in the laboratory animal unit of the Biological Faculty, M.V. Lomonosov Moscow State University with the access to food and water *ad libitum*. To obtain the offspring, sexually mature male and female rats were placed together for 4 days, pregnant females were housed individually 3–4 d before the expected delivery. The next day after birth, the litters were reduced to 8 pups. Experiments were carried out on male rats aged 11–15 d. In total, 50 pups (offspring of 20 females) with body weight 29 ± 1 g were used in the study. Pups were killed by decapitation.

2.2. Measurement of mRNA expression levels in arterial tissue by qPCR

Measurement of mRNA expression levels in arterial tissue by qPCR was performed as previously described [23]. Briefly, saphenous arteries were isolated, carefully cleaned from surrounding tissue and kept in RNAlater solution (cat. number 76106, Qiagen) at −20 °C pending further procedures. Each tissue sample included two arterial segments from young rats.

Arteries were homogenized in RLT lysis buffer (cat. number 79216, Qiagen) containing 1% β-mercaptoethanol and proteinase K (20 mg/ml, MP Biomedicals, United States). RNA was extracted using the ExtractRNA kit (cat. number BC032, Evrogen, Russia) according to the manufacturer's instructions and then processed with DNase I (cat. number EN0525, Fermentas, 1000 U/ml). The RNA concentration was measured by a NanoDrop 1000 (Thermo Scientific, United States), and then all samples were diluted to equal concentration 35 ng/µl. Reverse transcription was performed using the MMLV RT kit (cat. number SK021, Evrogen, Russia) according to the manufacturer's manual. qPCR was run in the Bio-Rad CFX 96 Real-Time PCR System (Bio-Rad, United States), using qPCRmix-HS SYBR (cat. number PK147L, Evrogen, Russia). Primers used in this study were synthesized by Evrogen (Russia), their sequences are listed in Table 1. The mRNA expression level was calculated as E^(-Cq), where E is the primer efficiency and Cq is the cycle number corresponding to the maximum of the second derivative of the fluorescence curve. Primer efficiency was identified using the LinRegPCR Software [24]. All E values were close to 2.0. mRNA expression levels of investigated genes were normalized to mRNA expression level of housekeeping gene *Actb*, detected in the same sample.

2.3. Experiments on the isolated arteries (wire myography)

The saphenous arteries were carefully cleaned from surrounding tissue in PSS for vessel dissection (PSS I, the composition, in mM: NaCl, 145; KCl, 4.5; $CaCl_2$, 0.1; $MgSO_4$, 1.0; NaH_2PO_4, 1.2; EDTA, 0.025; HEPES, 5.0; pH = 7.4), cut into 2-mm-long segments, and mounted in a wire myograph (410 A or 620 M, DMT A/S, Denmark) to measure isometric force. Experiments were carried out on endothelium-denuded arteries. The endothelium was removed mechanically using a rat whisker. During the subsequent experiments, arterial segments were kept in PSS II (the composition, in mM: NaCl, 120; $NaHCO_3$, 26; KCl, 4.5; $CaCl_2$, 1.6; $MgSO_4$, 1.0; NaH_2PO_4, 1.2; D-glucose, 5.5; EDTA, 0.025; HEPES, 5). The solution in myograph chambers was heated to 37 °C and continuously bubbled with 5% CO_2 + 95% O_2 to maintain pH at 7.4. Data were recorded at 10 Hz sampling rate using an analogue-to-digital converter (E14-140M, L-CARD, Russia) and the PowerGraph 3.3 software (DISoft, Russia). Then the normalization procedure was carried out to stretch each arterial segment to 0.9 × d_{100} (90% of the inner diameter it would have at a transmural pressure of 100 mmHg), corresponding to maximum active force development [14,25].

Table 1. Gene specific primers used in the study.

Gene name	Forward	Reverse
Nox1	GGCACAGTCAGTGAGGATGTC	GCTTGTTGTGTGCACGCTGG
Nox2	CTGCCAGTGTGTCGGAATCT	ACACACCACTCCACGTTGAA
Nox3	TATCCAGTGCCCATCCATCT	GCCTTCAGTAACGCCTCTGT
Nox4	TTGGTGAACGCCCTGAACTT	TACCACCACCATGCAGACAC
Duox1	AGCTGTGGCCTGGATCCTCC	GACCCTGTTGCTAAGGTGTCG
Duox2	CCTGGGCGCTCTGTTGACTGG	GACAGCGTCGCTTAGCAGGCG
Actb	CAGGGTGTGATGGTGGGTATGG	AGTTGGTGACAATGCCGTGTTC

At the beginning of each experiment, all vessels were exposed to a standard startup procedure. This included: (a) application of noradrenaline (10 µM); (b) application of acetylcholine (10 µM) on the top of contraction induced by methoxamine (agonist of α_1-adrenoceptors, 1 µM), to confirm the removal of the endothelium (by the absence of a relaxation response to acetylcholine); (c) application of methoxamine (10 µM). After each step, segments of arteries were washed three times with PSS II; the total washing time was 15 min.

The experimental protocol included two sequential concentration–response relationships to methoxamine (concentration range from 0.01 to 100 µM). The first relationship was started 20 min after the end of the activation procedure. After washout, four arterial segments were incubated for 20 min with the following substances: (1) pan-inhibitor of NAPDH oxidase (VAS2870, 10 µM); (2) an inhibitor/blocker of potential target of NADPH oxidase derived ROS; (3) the combination of VAS2870 and an inhibitor/blocker; (4) an equivalent volume of the solvent and/or inactive analogue of an inhibitor. Then, without changing the incubation solution, the second concentration–response relationship to methoxamine was obtained. We applied Y27632 (3 µM) to inhibit Rho-kinase, GF109203X (10 µM) to inhibit PKC, PP2 (10 µM) to inhibit Src-kinase and nimodipine (0.1 µM) or verapamil (0.1 µM) to block LTCC. PP3 (10 µM) was used as inactive analogue of Src-kinase inhibitor PP2.

To calculate active force values at each methoxamine concentration, the force value at the fully relaxed state (obtained in PSS I after the normalization procedure) was subtracted from all recorded values. All active force values obtained during the second concentration–response relationship were expressed as the percentage of the maximum active force developed during the respective first concentration–response relationship.

2.4. Evaluation of $O_2^{\bullet-}$ production by lucigenin-enhanced chemiluminescence

Two Lum-100 chemiluminometers were used in the experiments, as described previously [14]. Briefly,

arterial samples were mounted on stainless steel hooks and then placed on the bottom of the 5 ml round bottom polystyrene tubes (inner diameter of 10 mm) containing 1 ml of physiological salt solution for myograph experiments (PSS II). Each arterial sample consisted of two saphenous arteries. During the experiment, the solution in the tube was kept at 37 °C and aerated with a gas mixture (95% O_2 + 5% CO_2) supplied by a peristaltic pump (Ismatec ISM834C, Switzerland) from low-pressure compliant balloon. The gas mixture entered the tube with arterial sample through a capillary, the tip of which was positioned so that the bubbles did not disturb the sample.

In the beginning of each experiment, standard activation procedures were performed by addition of (1) KCl 60 mM, (2) methoxamine (10 μM) and (3) acetylcholine (10 μM) to the preparations, the duration of each exposure was 5 min. Washout procedure was performed after each step by placing preparations into preheated PSS II for 5 min three times; the total washing time was 15 min. To evaluate $O_2^{\bullet-}$ production, lucigenin (20 μM) was added to the tubes. The experimental protocol included two NADPH (100 μM) applications for each channel (in the presence of 10 μM of methoxamine). The first NADPH application (lasting 10 min) started 20 min after the end of the activation procedure. After that the samples were washed as described above. To study the effect of LTCC blockade on basal and NADPH-induced $O_2^{\bullet-}$ production, one arterial preparation was incubated for 15 min with nimodipine (0.1 μM) and the other one - with an equivalent volume of the solvent (DMSO, 1 μl per 1 ml of PSS II); then NADPH was added again for 10 min to both samples.

The level of chemiluminescence was continuously recorded in relative luminescence units (RLU) at 1 Hz sampling rate using PowerGraph 3.3 software (DISoft, Russia). When processing the results, we averaged the data in three intervals: background luminescence (5 min before lucigenin addition, in the beginning of the experiment), basal $O_2^{\bullet-}$ production in the presence of nimodipine or DMSO (5 min, starting 10 min after lucigenin addition) and NADPH-stimulated $O_2^{\bullet-}$ production in the presence of nimodipine or DMSO (9 min, starting 1 min after NADPH addition). Then the background value was subtracted from the other two and the data were normalized to wet tissue weight (RLU/mg).

2.5. Drugs

Noradrenaline, acetylcholine, methoxamine and lucigenin (cat. number M8010) (all dissolved in H_2O) were obtained from Sigma (United States). VAS2870 (cat. number SML0273, dissolved in DMSO) and GF109203X (cat. number G2911, dissolved in DMSO) were obtained from Sigma (United States), NADPH (cat. number 00616, dissolved in H_2O) was obtained from ChemImpex. PP2 and PP3 (cat. numbers A8216 and B7190, respectively, both dissolved in DMSO) were obtained from ApexBio (United States). Nimodipine (cat. number CA-211, dissolved in DMSO) and verapamil (cat. number CA-215, dissolved in H_2O) were obtained from Biomol (Germany).

2.6. Statistical data analysis

Statistical analysis was performed using GraphPad Prism 8.0. The normality of data distribution was confirmed using the Shapiro–Wilk test, data are presented as the mean and standard error of the mean. Statistical analysis was performed using one-way ANOVA (with Tukey's multiple comparisons test) and two-way Repeated Measures ANOVA (with Tukey's multiple comparisons test). Differences were accepted as statistically significant if the P value was less than 0.05, n represents the number of animals.

3. Results

3.1. mRNA expression of NADPH oxidase isoforms in saphenous artery of young rats

First, we evaluated the mRNA expression pattern of different NADPH oxidase isoforms in saphenous artery of young rats. Catalytic subunits of *Nox2, Nox4, Duox1* and *Duox2* were detected, wherein mRNA of *Nox2* was the most abundant (Figure 1). *Nox1* and *Nox3* mRNAs were not observed (Figure 1).

3.2. Effects of inhibition of NADPH oxidase together with Rho-kinase or PKC or Src-kinase on arterial contractile responses

Our next step was to examine if ROS, produced by NADPH oxidase, potentiate methoxamine-induced vasocontraction by activation of signaling pathways that increase calcium sensitivity of the contractile apparatus: Rho-kinase, PKC and Src-kinase.

In the first series of experiments, pan-inhibitor of NADPH oxidase VAS2870 significantly reduced arterial contractile responses to methoxamine (Figure 2). Inhibitor of Rho-kinase Y27632 attenuated methoxamine-induced vasocontraction as well (Figure 2). Importantly, the effect of VAS2870 persisted in the presence of Rho-kinase inhibitor (Figure 2).

Similarly, VAS2870 weakened methoxamine-induced vasocontraction in the second series of experiments (Figure 3). Inhibition of PKC resulted in the attenuation of arterial contractile responses to methoxamine (Figure 3). However, the effect of VAS2870 persisted after inhibition of PKC (Figure 3).

In the third series of experiments, inhibitor of NADPH oxidase and Src-kinase inhibitor PP2 both reduced arterial contractile responses to methoxamine (Figure 4). However, the combination of PP2 and VAS2870 resulted in more pronounced weakening of methoxamine-induced vasocontraction in comparison to the effect of PP2 alone (Figure 4).

Therefore, inhibition of Rho-kinase, PKC and Src-kinase did not abolish the effect of NADPH oxidase inhibitor VAS2870, indicating that these signaling pathways are not the key targets of NADPH oxidase-derived ROS in saphenous arteries of 11–15-day-old rat pups.

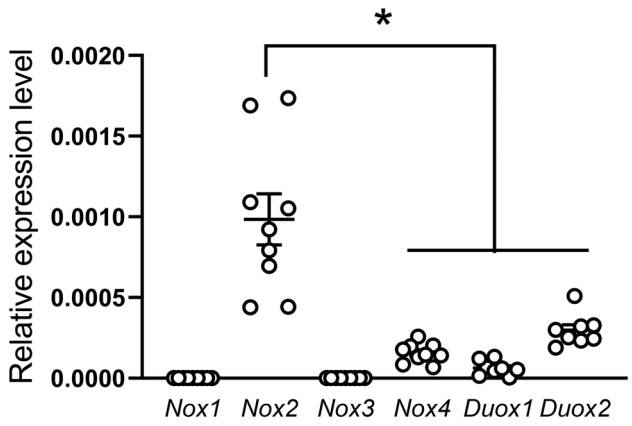

Figure 1. mRNA Expression of *Nox1-4* and *Duox1-2* catalytic subunits in saphenous artery of young rats. Data are normalized to mRNA expression level of beta-actin (*Actb*). The number of samples in each group is at least 8. Data are presented as mean and SEM. *$p < .05$ (one-way ANOVA with Tukey's multiple comparisons test).

3.3. Effects of inhibition of NADPH oxidase together with LTCC blockade on arterial contractile responses

According to the literature, ROS, produced by NADPH oxidase, may activate LTCC and, therefore, promote vasocontraction [21,26]. To test if NADPH oxidase derived ROS contribute to methoxamine-induced contraction by activation of these channels, we performed the series of experiments with the use of LTCC blockers: nimodipine and verapamil. As Figure 5 shows, when added separately all VAS2870, nimodipine and verapamil weakened methoxamine-induced contraction. Importantly, VAS2870 did not reduce further contractile responses to methoxamine in the presence of nimodipine or verapamil (Figure 5). Therefore, LTCC are involved into procontractile effect of NADPH oxidase derived ROS in saphenous artery of young rats.

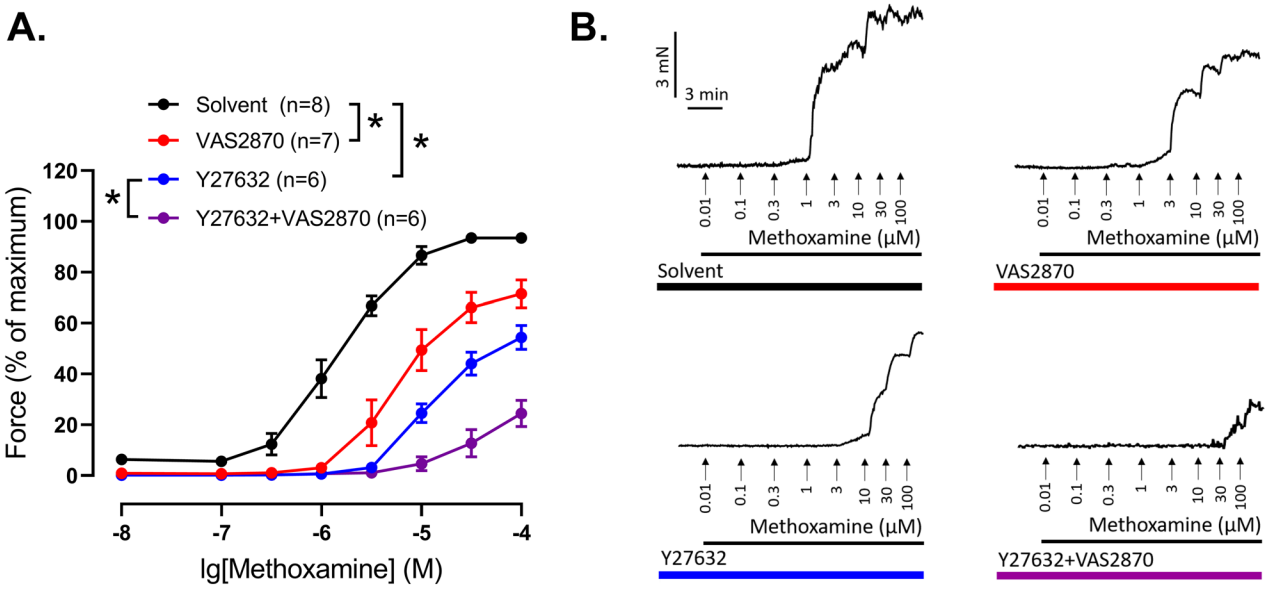

Figure 2. Rho-kinase inhibition does not abolish the effect of NADPH oxidase inhibitor. Contractile responses of the saphenous artery to methoxamine in the presence of solvent or pan-NADPH oxidase inhibitor VAS2870 (10 μM) or Rho-kinase inhibitor Y27632 (3 μM) or Y27632 together with VAS2870 (a). Fragments of original recordings of the experiment (b). The numbers in brackets indicate the number of animals in the group. *$p < .05$ (two-way Repeated Measures ANOVA followed by Tukey's multiple comparisons test).

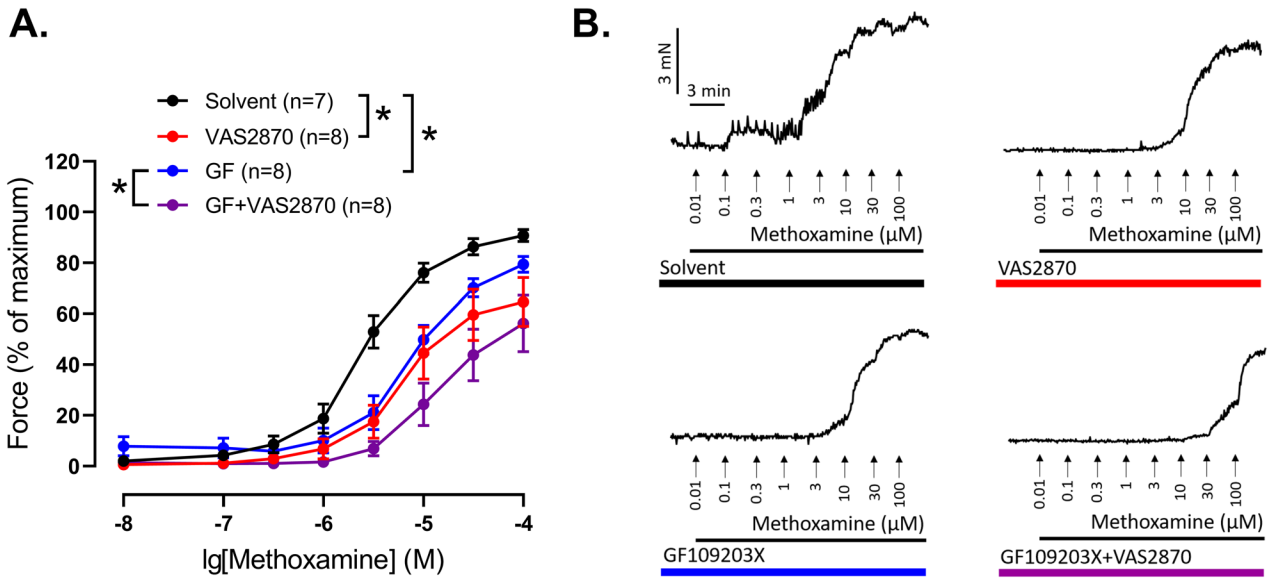

Figure 3. Protein kinase C inhibition does not abolish the effect of NADPH oxidase inhibitor. Contractile responses of the saphenous artery to methoxamine in the presence of solvent or pan-NADPH oxidase inhibitor VAS2870 (10 μM) or protein kinase C inhibitor GF109203X (GF, 10 μM) or GF109203X together with VAS2870 (a). Fragments of original recordings of the experiment (b). The numbers in brackets indicate the number of animals in the group. *$p < .05$ (two-way Repeated Measures ANOVA followed by Tukey's multiple comparisons test).

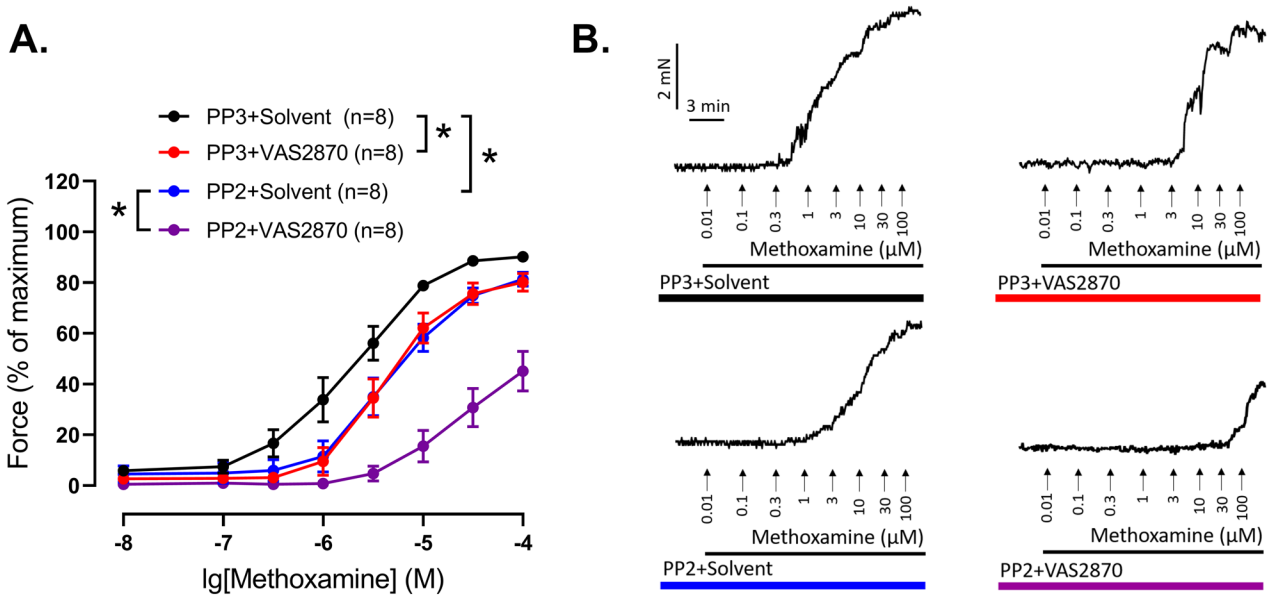

Figure 4. Src-kinase inhibition does not abolish the effect of NADPH oxidase inhibitor. Contractile responses of the saphenous artery to methoxamine in the presence of PP3 (10 μl, negative control for PP2) and solvent or PP3 and pan-NADPH oxidase inhibitor VAS2870 (10 μM) or Src-kinase inhibitor PP2 (10 μM) and solvent or PP2 together with VAS2870 (a). Fragments of original recordings of the experiment (b). The numbers in brackets indicate the number of animals in the group. *$p < .05$ (two-way Repeated Measures ANOVA followed by Tukey's multiple comparisons test).

3.4. Effects of LTCC blockade on $O_2^{\bullet-}$ production in saphenous artery of young rats

In order to ensure that NADPH oxidase derived ROS activate LTCC, and not calcium that entered through LTCC activate NADPH oxidase, we performed measurements of $O_2^{\bullet-}$ production with the use of lucigenin-enhanced chemiluminescence. Blockade of LTCC by nimodipine did not affect either basal $O_2^{\bullet-}$ production, or NADPH-induced $O_2^{\bullet-}$ production (Figure 6). Therefore, results of chemiluminescence measurement

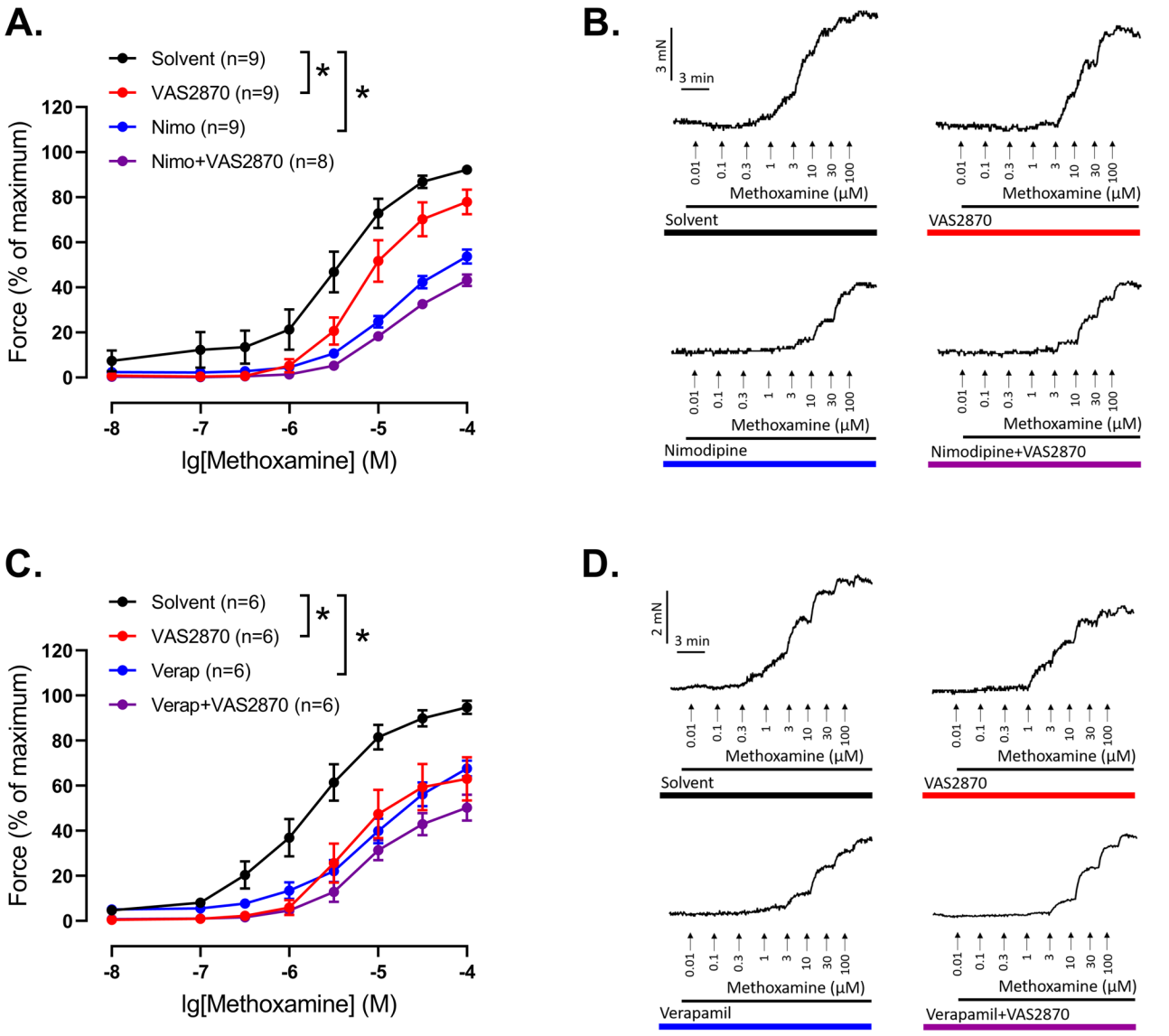

Figure 5. LTCC blockade abolishes the effect of pan-inhibitor of NADPH oxidase. Contractile responses of the saphenous artery to methoxamine in the presence of solvent or NADPH oxidase inhibitor VAS2870 (10 μM) or LTCC blocker nimodipine (nimo, 0.1 μM) or verapamil (verap, 0.1 μM) or nimodipine/verapamil together with VAS2870 (a, c). Fragments of original recordings of the experiments (b, d). The numbers in brackets indicate the number of animals in the group. *$p < .05$ (two-way Repeated Measures ANOVA followed by Tukey's multiple comparisons test).

indicate that calcium entering through LTCC does not affect basal and NADPH-induced $O_2^{\cdot-}$ production.

4. Discussion

4.1. mRNA of Nox2 is the most abundant NADPH oxidase isoform in saphenous artery of young rats

According to the literature, mRNAs of *Nox1*, *Nox2* and *Nox4* are most abundant among various arteries [27–30]. Present data, like our data obtained earlier, demonstrate that saphenous artery of young rats contains mRNAs of *Nox2* and *Nox4*, while mRNAs of *Nox1* and *Nox3* are not expressed [14].

Of note, the expression of *Duox1* and *Duox2* in vascular tissue was assessed only in a few studies [31,32], and their expression in the arteries of early postnatal organism was not demonstrated previously. Notably, the key pharmacological tool used in the present study – VAS2870, inhibits all isoforms of NADPH oxidase, including dual oxidases [33–35]. Therefore, it was important to evaluate the abundance of *Duox1* and *Duox2* in saphenous artery of young rats. For the first time we demonstrated that mRNAs of *Duox1* and *Duox2* catalytic subunits are expressed in saphenous artery of young rats. However, the relative expression levels of *Duox1* and *Duox2* as well as *Nox4* genes were significantly lower in comparison to *Nox2*. In other

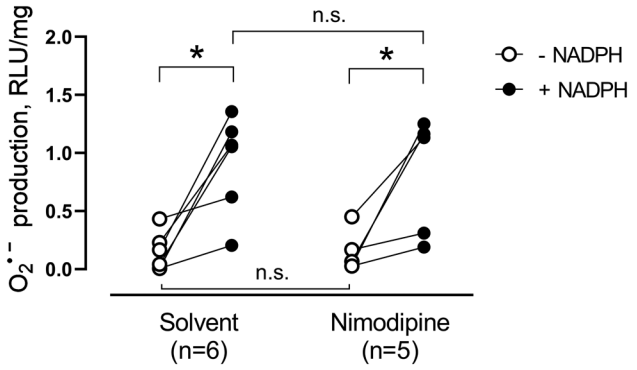

Figure 6. LTCC blocker nimodipine (0.1 μM) does not affect basal (–NADPH) and NADPH-stimulated $O_2^{\bullet-}$ production (+NADPH) in saphenous arteries of young rats. The numbers in brackets indicate the number of animals in the group. *$p < .05$ (two-way ANOVA followed by Tukey's multiple comparisons test), n.s.: not significant.

words, among all isoforms of NADPH oxidase, the catalytic subunit of the second isoform is the most abundant in saphenous artery of young rats.

Importantly, previously we have demonstrated that the inhibitor of the second isoform of NADPH oxidase weakened methoxamine-induced contraction of saphenous arteries of young rats [14], indicating that this NADPH oxidase isoform is functionally important.

4.2. ROS, produced by NADPH oxidase, promote vasocontraction in response to methoxamine regardless activity of Rho-kinase, PKC or Src-kinase in saphenous artery of young rats

In confirmation of our recently published data [14], we observed a significant weakening of methoxamine-induced contraction of early postnatal rat arteries by inhibition of NADPH oxidase with the use of VAS2870. In this study, we explored the mechanisms of such procontractile influence of NADPH oxidase derived ROS, considering the peculiarities of vascular tone regulation during early postnatal ontogenesis.

Literature data demonstrate, that ROS may promote contraction of adult rat aorta and pulmonary artery, by activation of Rho-kinase [4,5,36]. In neonatal period, ROS contribute to contraction of human, rabbit and chicken ductus arteriosus at least partially by activation of Rho-kinase [37,38]. Notably, functional impact of Rho-kinase to the regulation of vascular tone during early postnatal period is especially high, which was previously demonstrated in saphenous artery of 1-week old rats [18,19]. Surprisingly, according to our data, inhibition of Rho-kinase activity by Y27632 did not abolish the effect of NADPH oxidase derived ROS, showing that Rho-kinase is not the main target of their procontractile influence. In accordance with this, ROS effects on vascular tone are not always associated with Rho-kinase activity, for example, they can cause contraction of adult pulmonary arteries regardless of Rho-kinase [4,39]. The involvement of Rho-kinase in contraction was shown to depend on the type of ROS: $O_2^{\bullet-}$ [5], but not H_2O_2 [39] action leads to the activation of Rho-kinase. As mentioned above, in the saphenous artery of rat pups, the key functional contribution to the regulation of tone is made by the second isoform of NADPH oxidase, which produces predominantly $O_2^{\bullet-}$ [40]. However, we cannot claim that it is $O_2^{\bullet-}$, not H_2O_2, that acts further as a signaling molecule, since the dismutation of $O_2^{\bullet-}$ into H_2O_2 occurs very rapidly.

It is known, that ROS produced by NADPH oxidase may activate other kinases, such as Src-kinase [6] and PKC [21,41], contributing to vasocontraction in response to different stimuli. Procontractile role of Src-kinase and PKC was demonstrated in a number of studies [18,42, 43], and was confirmed in the present study by the weakening of methoxamine-induced contractile responses of saphenous arteries from young rats after incubation with PP2 and GF109203X, respectively. The pathways through which Src-kinase and PKC mediate ROS-induced contraction include those involving alterations of $[Ca^{2+}]_{in}$ (either directly *via* activation of Ca^{2+} channels or indirectly *via* inhibition of potassium channels), and those independent of changes in $[Ca^{2+}]_{in}$ [6,22,42]. However, our data demonstrate that neither Src-kinase nor PKC are the main targets of procontractile influence of NADPH oxidase derived ROS. Probably, the involvement of these kinases into the procontractile influence of ROS, produced by NADPH oxidase, may depend on several factors: the animal species, the vascular region, and, importantly, the stage of development.

4.3. ROS, produced by NADPH oxidase, promote vasocontraction in response to methoxamine by activation of LTCC in saphenous artery of young rats

LTCC are major sources of calcium influx into arterial smooth muscle cells, and therefore, key participants of the regulation of vascular tone [44]. Thus, it is not surprising that in the present study methoxamine-induced contraction of saphenous artery of early postnatal rats was significantly reduced after incubation with LTCC blockers nimodipine or verapamil.

Importantly, numerous studies demonstrate the involvement of LTCC into ROS-induced vasocontraction. For example, inhibitory effects of LTCC blockade on

contraction induced by exogenous ROS as well as endogenous NADPH oxidase derived ROS were demonstrated in aorta [10], cerebral arteries [21], renal afferent arterioles [11] of rats, in mesenteric arteries of mice [45] and in bovine pulmonary artery smooth muscle cells [26]. In accordance with this, our data show that NADPH oxidase inhibitor did not cause an additional reduction in contractile responses under LTCC blockers nimodipine or verapamil, indicating that procontractile influence of NADPH oxidase derived ROS is due to activation of LTCC in saphenous artery of young rats.

Of note, several participants of ROS-dependent regulation of vascular tone can serve not only as downstream targets of NADPH oxidase derived ROS, but, at the same time, be upstream molecules, regulating the activity of NADPH oxidase and, consequently, ROS production [9,26,46]. It is known that an increase of $[Ca^{2+}]_{in}$ is a primary mechanism of dual oxidase activation [47]. Besides that, some isoforms of NADPH oxidase, including the second isoform, can be activated by Ca^{2+} indirectly [48,49]. We demonstrated that blockade of LTCC with the use of nimodipine did not affect basal and NADPH-induced $O_2^{\bullet-}$ production in saphenous artery of young rats. Importantly, a significant part of NADPH-stimulated $O_2^{\bullet-}$ in saphenous artery of young rats is produced by NADPH oxidase [14]. Therefore, we suggest that NADPH oxidase derived ROS activate LTCC, but not calcium entering through LTCC activates NADPH oxidase.

The mechanism of such ROS influence on LTCC remains open. ROS-dependent stimulation of LTCC may be indirect *via* oxidative activation of protein kinases (such as Src-kinase or PKC) that activate the channel by phosphorylation [44]. However, our experiments with the use of Src-kinase and PKC inhibitors indicate that these kinases play not leading role in the procontractile influence of NADPH oxidase derived ROS in response to methoxamine in saphenous artery of young rats. Therefore, activation of LTCC *via* their phosphorylation by Src-kinase or PKC seems unlikely. One more indirect way of ROS-dependent stimulation of LTCC may realize through inhibition of potassium channels, depolarization of the plasma membrane and thus opening of LTCC. Indeed, ROS, including those produced by NADPH oxidase, were shown to promote contraction of ductus arteriosus [50] and pulmonary artery smooth muscle cells [26], at least partly, by the inhibition of Kv channels. Taking into account very high anticontractile contribution of Kv channels to the regulation of vascular tone in early postnatal ontogenesis [51,52], it can be assumed that ROS produced by NADPH oxidase promote vasocontraction in response to methoxamine by inhibition of Kv channels and, therefore, activation of LTCC. Finally, LTCC activation by ROS may occur directly - through oxidation of cysteine residues [53,54], which LTCC pore-forming α1c subunit is especially rich in (55].

At first glance, such ROS-dependent mechanism of vascular tone control in neonates seems unlikely, since LTCC are sparse in undifferentiated vascular smooth muscle cells [56,57] and, accordingly, Ca^{2+}-dependent control of arterial contraction in neonates is weak [15]. However, by the age of two weeks, an increase in $[Ca^{2+}]_{in}$ during activation of membrane receptors (including α_1-adrenoceptors) is already rising [15]. Importantly, LTCC-dependent pattern of $[Ca^{2+}]_{in}$ fluctuations in smooth muscle cells affects not only their tone, but also expression of contractile phenotype marker genes/proteins [58,59]. Thus, by stimulating the activity (our study), as well as the expression of LTCC [60], NADPH oxidase derived ROS can accelerate the differentiation of smooth muscle cells into a contractile phenotype.

4.4. Limitations of the study

The present study has several limitations. First, NADPH oxidase derived ROS would have a stronger effect on LTCC if ROS were produced in close proximity to LTCC. However, the spatial relationship between NADPH oxidase and LTCC in smooth muscle cells of early postnatal rat saphenous artery was not addressed in our study. Second, we did not measure Ca^{2+} influx through LTCC and, therefore, have no direct evidence of its modulation by NADPH oxidase produced ROS.

5. Conclusions

In conclusion, this study reveals the mechanism of a highly pronounced procontractile influence of NADPH oxidase derived ROS in the peripheral arteries of early postnatal rats [14]. Despite a significant contribution of Ca^{2+}-sensitization in arteries at early postnatal period [18,19], our novel findings demonstrate that Rho-kinase, PKC and Src-kinase are not the main targets of NADPH oxidase derived ROS. At the same time, we demonstrated that Ca^{2+}-dependent mechanism, namely, activation of LTCC, mediates the procontractile influence of NADPH oxidase derived ROS in arteries of early postnatal rats.

Our data complement and develop knowledge about important functional role of ROS in the cardiovascular system in early postnatal ontogenesis [3,14,50]. Notably, high production of ROS and further activation of LTCC are often observed during the development of hypertension-related disorders in adulthood [60]. The

fact that similar mechanism participates in normal vasoregulation at early stages of development, further demonstrates the physiological features of the immature organism. The latter, in turn, indicates the need of special therapeutic approaches in newborns, including those intended to treat "oxygen radical disease in newborn" [61–63].

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Authors contributions

Conceptualization A.A.S., D.K.G., O.S.T.; Funding acquisition A.A.S.; Investigation V.S.S., M.A.K., Y.A.M., A.A.S.; Writing (original draft) A.A.S.; Writing (review & editing) A.A.S., D.K.G., O.S.T.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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