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Research Article

# Neuronal nitric oxide synthase-derived hydrogen peroxide effect in grafts used in human coronary bypass surgery

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Recently, $H_2O_2$ has been identified as the endothelium-dependent hyperpolarizing factor (EDHF), which mediates flow-induced dilation in human coronary arteries. Neuronal nitric oxide synthase (nNOS) is expressed in the cardiovascular system and, besides NO, generates $H_2O_2$. The role of nNOS-derived $H_2O_2$ in human vessels is so far unknown. The present study was aimed at investigating the relevance of nNOS/$H_2O_2$ signaling in the human internal mammary artery (IMA) and saphenous vein (SV), the major conduits used in coronary artery bypass grafting. In the IMA, but not in the SV, ACh (acetylcholine)-induced vasodilatation was decreased by selective nNOS inhibition with TRIM or Inhibitor 1, and by catalase, which specifically decomposes $H_2O_2$. Superoxide dismutase (SOD), which generates $H_2O_2$ from superoxide, decreased the vasodilator effect of ACh on SV. In the IMA, SOD diminished phenylephrine-induced contraction in endothelium-containing, but not in endothelium-denuded vessels. Importantly, while exogenous $H_2O_2$ produced vasodilatation in IMA, it constricted SV. ACh increased $H_2O_2$ production in both sets of vessels. In the IMA, the increase in $H_2O_2$ was inhibited by catalase and nNOS blockade. In SV, $H_2O_2$ production was abolished by catalase and reduced by nNOS inhibition. Immunofluorescence experiments showed the presence of nNOS in the vascular endothelium and smooth muscle cells of both the IMA and SV. Together, our results clearly show that $H_2O_2$ induced endothelium-dependent vascular relaxation in the IMA, whereas, in the SV, $H_2O_2$ was a vasoconstrictor. Thus, $H_2O_2$ produced in the coronary circulation may contribute to the susceptibility to accelerated atherosclerosis and progressive failure of the SV used as autogenous graft in coronary bypass surgery.

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## Introduction

The internal mammary artery (IMA) and the saphenous vein (SV) are the main vascular tissues used as grafts in coronary artery bypass grafting (CABG) [1]. However, when grafted into the coronary circulation, SV grafts undergo profound remodeling, which compromises their long-term viability [2,3]. Moreover, vasospasm of the grafts following CABG surgery is a major problem and may cause perioperative and late failure of bypass conduits [4]. The integrity of the vascular endothelium is also a key factor in determining the fate of SV and IMA grafts following implantation [5].

In the present context, an important property of vascular endothelium is the release of vasodilator metabolites such as nitric oxide (NO) [6], prostacyclin ($PGI_2$) [7] and cytochrome P450 metabolites

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of arachidonic acid [8], which is stimulated by the shear stress of blood flow. More recently, hydrogen peroxide ($H_2O_2$) has emerged as the endothelium-derived hyperpolarizing factor (EDHF) that mediates flow-induced dilation of human coronary arterioles (HCA) [9–11]. Moreover, while NO and $PGI_2$ seem to play a more important role in the HCA of healthy patients, the role of $H_2O_2$, as a vasodilator factor in HCA is more prominent in certain disease states such as in coronary artery disease [9].

$H_2O_2$ has also been considered as the EDHF responsible for agonist-induced dilation in porcine and human [10] coronary micro vessels, and in murine and human mesenteric arteries [12]. However, it is important to note that, apart from vasodilation [13–16], $H_2O_2$ may cause vasoconstriction [17–19], depending on the vessel or experimental condition. It was, therefore, possible that the effects of $H_2O_2$ on the IMA could differ from its effects on the SV, especially when grafted into the vascular bed.

Neuronal nitric oxide synthase (nNOS) is expressed in the vascular endothelium [20,21] and in smooth muscle cells [22]. The activity of this isoform of NOS is relevant to the physiological modulation of myogenic tone [23], systemic arterial pressure [24] and blood flow [25,26]. We have shown that, in addition to NO, nNOS generates $H_2O_2$, which in the mouse aorta and small mesenteric artery contributes to endothelium-dependent vascular relaxation [21,27,28]. However, the effect of $H_2O_2$ derived from nNOS in human vascular tissue is not known.

Given the importance of $H_2O_2$ in the coronary circulation and the lack of information available regarding the role of nNOS and $H_2O_2$ in human vascular function, the present study investigated the role of nNOS-derived $H_2O_2$ in the regulation of vascular function in the IMA and SV of patients undergoing coronary bypass graft. A particular focus was on any differences between the IMA and SV in their responses to endogenously generated $H_2O_2$.

# Experimental

## Assessment of vascular function

We used IMA and SV segments from human subjects undergoing coronary artery bypass grafting ($n = 148$). All the experimental procedures were carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association and approved by the ethics committee of the Universidade Federal de Minas Gerais (protocol CAAE: 31961214.6.0000.5149), and informed consent was obtained from all participants. The mean age of the patients was $61.3 \pm 11.9$ years. After the surgical procedure, the vessels were immediately transferred to the laboratory in cold (4 °C) Krebs–Henseleit solution (the composition in mM was as follows: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1). IMA and SV (2–3 mm in length) were cut into rings and mounted in organ baths containing Krebs–Henseleit solution gassed with 5% $CO_2$ in $O_2$ at 37 °C and pH 7.4. The rings were stretched to an optimal load of 1.5 g for the IMA [29] and 4 g for the SV [30,31]. In some preparations, the endothelium was mechanically removed. Changes in mechanical activity were recorded isometrically by a force transducer (World Precision Instruments, Inc., Sarasota, FL, USA) connected to an amplifier–recorder (TBM-4 model; World Precision Instruments, Inc., USA) and to a personal computer equipped with an analogue-to-digital converter board (DI-720), using WinDaq Data Acquisition software (Dataq® Instruments, USA). Concentration–response curves were performed on each vascular ring in the presence or in the absence of specific drugs, as indicated.

Following a 60-minute stabilization period, all rings were challenged with 80 mM KCl to evaluate the maximal contraction possible for each ring. Endothelial integrity was tested with acetylcholine (ACh; 10 $\mu$M) in segments previously contracted with phenylephrine (1 $\mu$M). Relaxation greater than 60% and 20% was considered demonstrative of the functional integrity of the endothelium in the IMA [32] and SV, respectively [30,33,34]. After a 45-min washout period, vessels were pre-contracted with phenylephrine (1 $\mu$M; inducing 75% of 80 mM KCl maximal contraction) and when the preparations reached a plateau, concentration–response curves to ACh were performed. Then, the vessels were washed out until they returned to their optimal load and were incubated for 30 minutes with different drugs, and a second cumulative concentration–response curve for ACh was constructed and compared with the first. The drugs used were: the non-specific inhibitor of NOS L-$N^G$-nitroarginine methyl ester (L-NAME; 300 $\mu$M); the non-specific inhibitor of cyclooxygenase (COX) indomethacin (10 $\mu$M), catalase (2000 U/ml), which selectively decomposes $H_2O_2$ [35,36]; superoxide dismutase (SOD; 300 U/ml,) an enzyme that dismutes $O_2^-$ into $H_2O_2$ [37,38]; and the selective nNOS inhibitors 1-(2-trifluoromethylphehyl) imidazole (TRIM; 300 $\mu$M) and (4S)-N-(4-amino-5[aminoethyl]aminopentyl)-N′-nitroguanidine (Inhibitor 1; 5 $\mu$M). The concentrations of TRIM and Inhibitor 1 were chosen on the basis of their selectivity for nNOS over other isoforms [39–41]. In some experiments, cumulative concentration–response curves to exogenous $H_2O_2$ were performed in endothelium-denuded vessels pre-contracted with phenylephrine (1 $\mu$M). In a separate set of experiments, cumulative concentration–response curves to phenylephrine were constructed using the IMA in the presence and in the absence of SOD (300 U/ml).

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## Hydrogen peroxide production in the IMA and SV

Spectrofluorimetry was used to measure the ACh-induced $H_2O_2$ released in human IMA and SV. Small rings were placed in physiological salt solution (PSS) containing the $H_2O_2$ marker probe, 2′,7′ dichlorodihydrofluorescein-diacetate (5 $\mu$M DCF-DA) for 30 minutes and kept at 37°C in a dry bath. Subsequently, the vessels were rinsed in a solution of PSS without DCF-DA for 10 minutes and stimulated with ACh in the presence or absence of catalase (2400U/ml), $N^G$-nitro-L-arginine (L-NNA; 100 $\mu$M), TRIM (300 $\mu$M), Inhibitor 1 (5 $\mu$M), indomethacin (10 $\mu$M) or L-NNA + indomethacin. The assessment of the basal production of $H_2O_2$ was performed in vessels before the stimulation with ACh. For the measurement of $H_2O_2$, 100 $\mu$l of the perfusate was placed into a 96-well microplate (Axygen®) and the samples were read in a spectrofluorometer (fluoroscan Ascent FL Thermo-Cientific) at 485 nm excitation and 538 nm emission. The reaction proceeded at room temperature and in the dark. The values from each experiment were normalized to the dry weight of each IMA or SV ring.

## Immunolocalization of nNOS

Immunolocalization of nNOS in human IMA and SV was performed as previously described, with some modifications [42]. Briefly, fixed cryosections (10 $\mu$m) of IMA and SV were fixed in cold acetone for 15 minutes and rinsed in phosphate-buffered saline (PBS) wash buffer (1% BSA and 0.3% Triton X-100, in PBS). The blocking procedures (3% BSA and 0.3% Triton X-100 in PBS, 30 minutes) were performed to remove cross reactivity of secondary antibody with the alternating primary antibody. Slides were incubated with mouse anti-nNOS anti-body (1:50) overnight at 4°C followed by incubation with goat anti-mouse secondary antibody conjugated with Alexa Fluor 555 (1:500). The slices were mounted with DAPI/UltraCruz R Mounting Medium (Santa Cruz Biotechnology, Inc., CA, USA) and digital images were obtained using a fluorescence microscope (Axio Imager Zeiss 2 Apotome, Germain) using a standard filter with a 63× objective. Four fields per slide of endothelium and media layers were observed and images shown are representative of five patients. Immunostained sections were examined on an Apotome microscope, with excitation at 555 nm for nNOS (represented by images in red color), excitation at 488 nm for elastic laminae autofluorescence (represented by images in green color) and DAPI at 358 nm (represented by images in blue color).

## Statistical analysis

Data are expressed as the means ± SEM. Two-way ANOVA with Bonferroni's multiple comparisons post-test was used to compare concentration–response curves. One-way ANOVA followed by Newman-Keuls multiple-comparison was used in Figure 7. All statistical analyses were calculated using Prism 4.2 software (GraphPad) and were considered to be significant when $P < 0.05$.

## Drugs

ACh, phenylephrine, L-NAME, L-NNA, TRIM, indomethacin, catalase and SOD, were purchased from Sigma-Aldrich Inc, (St. Louis, MO, USA); Inhibitor 1 was purchased from Calbiochem (San Diego, CA, USA); $H_2O_2$ was purchased from Merck (Darmstadt, Hessen, Germany); the primary antibodies anti-PECAM-1 and anti-NOS1 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA); and DCF-DA probe, the secondary antibody conjugated with Alexa Fluor 555 was purchased from Invitrogen (Carlsbad, CA, USA).

# Results

## Vascular reactivity studies

A concentration-dependent vasodilator effect in response to ACh was observed only in endothelium-containing rings from IMA (Figure 1A) and SV (Figure 1B). Pre-treatment of vessels with L-NAME abolished the vasodilator response to ACh in IMA rings (Figure 2A). On the other hand, only a partial inhibition was observed for SV in the same conditions (Figure 2B). However, when SV was pre-treated with L-NAME + indomethacin, a full blockade of the vasodilator response was observed (Figure 2C).

We assessed the role of nNOS in the endothelium-dependent vasodilation in IMA and SV by pre-treatment of IMA and SV samples with two selective nNOS inhibitors, TRIM or Inhibitor 1. This pre-treatment significantly decreased ACh-induced relaxation (Figure 3A and 3B). However, no differences were observed in the relaxant responses to ACh in the presence of TRIM or Inhibitor 1 in SV (Figure 3C and 3D).

In addition to NO, vascular nNOS generates $H_2O_2$, which makes an important contribution to the total endothelium-dependent vascular relaxation [21,42]. We therefore assessed, here, the possible contribution of endogenous $H_2O_2$ to the ACh-induced vasodilatation in two types of human blood vessel using catalase to inactivate

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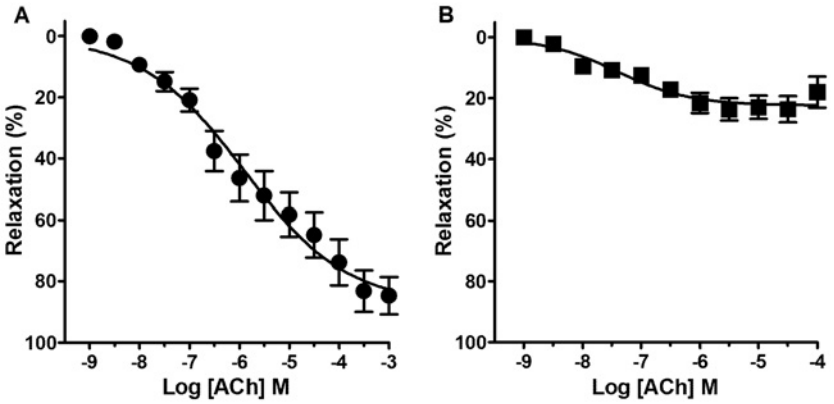


**Figure 1. Vasodilator effect of acetylcholine (ACh) in (A) endothelium-intact internal mammary artery (IMA) and (B) saphenous vein (SV)**

Results are shown as the mean ± S.E.M. $n = 10$ for IMA and $n = 8$ for SV.

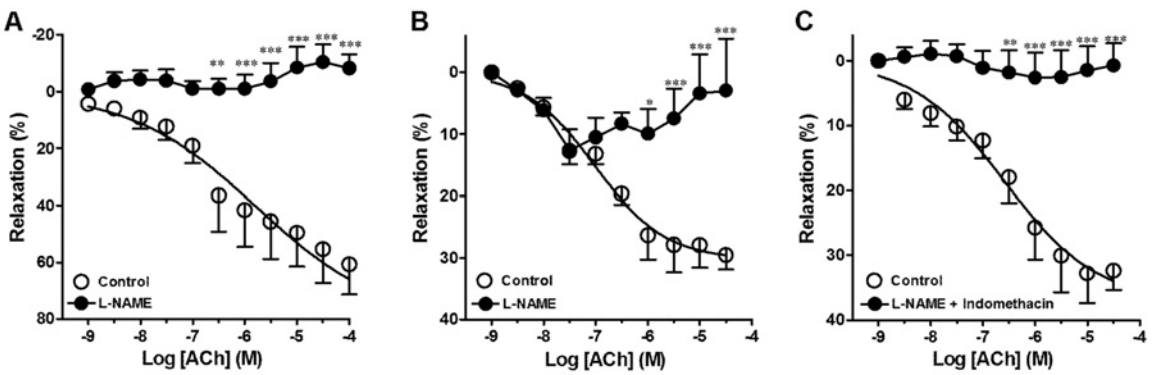


**Figure 2. Vasodilator effect of acetylcholine (ACh) in the absence (control) and in the presence of L-NAME (300 $\mu$M): (A) in endothelium-intact internal mammary artery (IMA) rings; (B) in endothelium-intact saphenous vein (SV); and (C) in the presence of L-NAME + indomethacin in endothelium-intact SV**

Results are shown as the mean ± S.E.M. A two-way ANOVA followed by Bonferroni's post-test was conducted. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$ for IMA and $n = 5$–8 for SV.

the $H_2O_2$ formed. In endothelium-containing IMA rings, pre-treated with catalase, ACh-induced vasodilatation was significantly decreased (Figure 4A). However, in samples of SV, catalase did not affect the vasorelaxant response to ACh (Figure 4B).

Another pathway to generating $H_2O_2$ is via the dismutation of superoxide by SOD. We therefore incubated IMA and SV samples with SOD before assaying the vasodilation induced by ACh. SOD markedly impaired the relaxant effect of ACh in endothelium-containing preparations of SV (Figure 5A). However, in the IMA, the pre-contraction with phenylephrine was not sustained and it was not possible to use the same protocol as with the SV. Therefore, we analyzed the effect of SOD on the concentration–response curve elicited by phenylephrine in IMA rings. As seen in Figure 5B, pre-treatment of the vessels with SOD decreased the constrictor response to phenylephrine in endothelium-containing preparations. Conversely, no change was observed when the same protocol was performed in IMA preparations after endothelium removal (Figure 5C).

To further compare the role of $H_2O_2$ in the IMA and SV, we carried out concentration–response curves to exogenous $H_2O_2$ in preparations where endothelium was mechanically removed. In endothelium-denuded IMA, exogenous $H_2O_2$ exerted a concentration-dependent vasodilator effect in vessels pre-contracted with phenylephrine (Figure 6A), whereas in similar preparations of SV, exogenous $H_2O_2$ induced a concentration-dependent contractile effect (Figure 6B). Inhibition of nNOS with TRIM did not change the $H_2O_2$ effects in both the IMA and SV (Figure 6A and 6B).

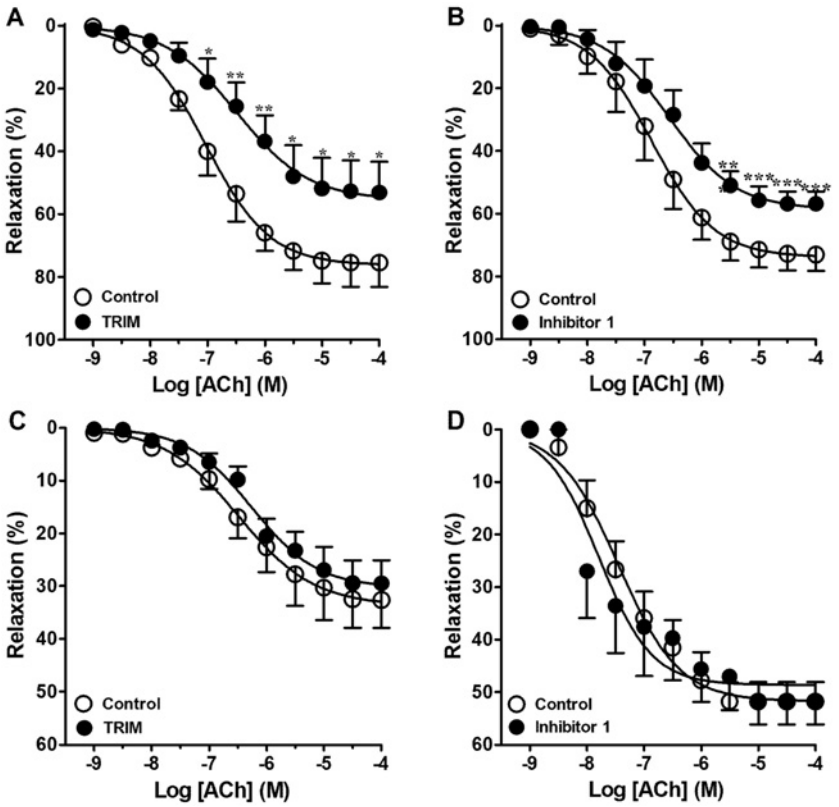


**Figure 3. Role of nNOS in the vasodilator effect of acetylcholine (ACh) in endothelium-intact rings from internal mammary artery (IMA) and saphenous vein (SV)**

Concentration–response curves to ACh in the absence (control) and in the presence of TRIM (300 $\mu$M) or Inhibitor 1 (5 $\mu$M) in IMA (**A** and **B**) and SV (**C** and **D**). Results are shown as the mean $\pm$ S.E.M. A two-way ANOVA followed by Bonferroni's post-test was conducted. *$P <$ 0.05, **$P <$ 0.01 and ***$P <$ 0.001; $n$ = 6–7 for IMA and $n$ = 6–11 for SV.

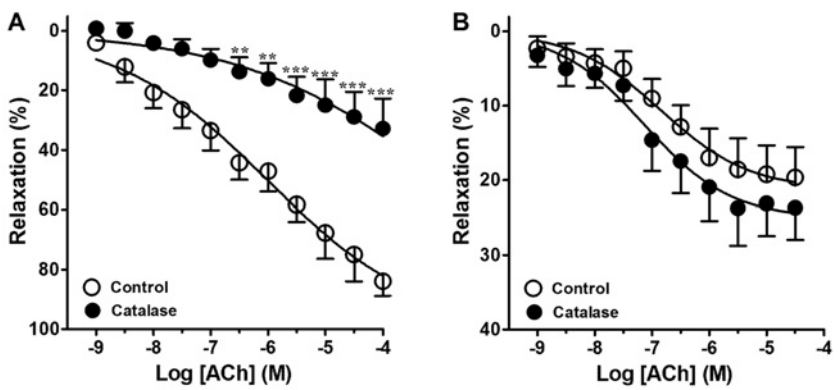


**Figure 4. Effect of catalase (2000 U/ml) on the vascular relaxation induced by acetylcholine (ACh) in endothelium-intact (A) internal mammary artery ($n$ = 6) and (B) saphenous vein ($n$ = 9)**

Results are shown as the mean $\pm$ S.E.M. A two-way ANOVA followed by Bonferroni's post-test was conducted. **$P <$ 0.01, ***$P <$ 0.001.

## Measurements of $H_2O_2$ produced in IMA and SV

For both the IMA and SV, the addition of ACh induced an increase in the production of $H_2O_2$ (Figure 7). When the vessels were pre-treated with catalase, no $H_2O_2$ could be detected in both the IMA and SV samples (Figure 7A and 7B). The non-selective NOS inhibitor L-NNA and the selective nNOS inhibitors, TRIM and Inhibitor-1, significantly

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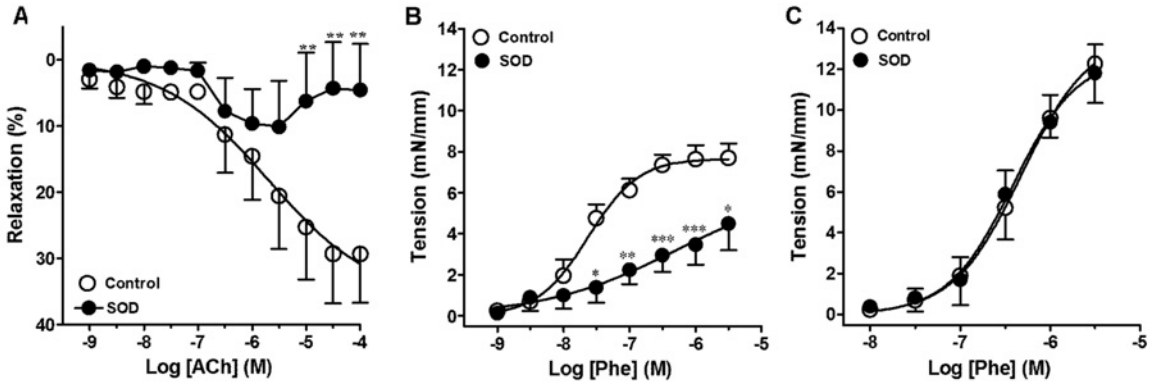


**Figure 5. Vasodilator effect of acetylcholine (ACh) in endothelium-intact saphenous vein (SV) in (A) the absence (control) and in the presence of SOD (300U/ml). Vasoconstrictor effect of phenylephrine (Phe) in (B) endothelium-intact and (C) endothelium-denuded internal mammary artery rings (IMA) in the absence (control) and in the presence of SOD (300 U/ml)**

Results are shown as the mean ± S.E.M. A two-way ANOVA followed by Bonferroni's post-test was conducted. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$ for both SV and IMA.

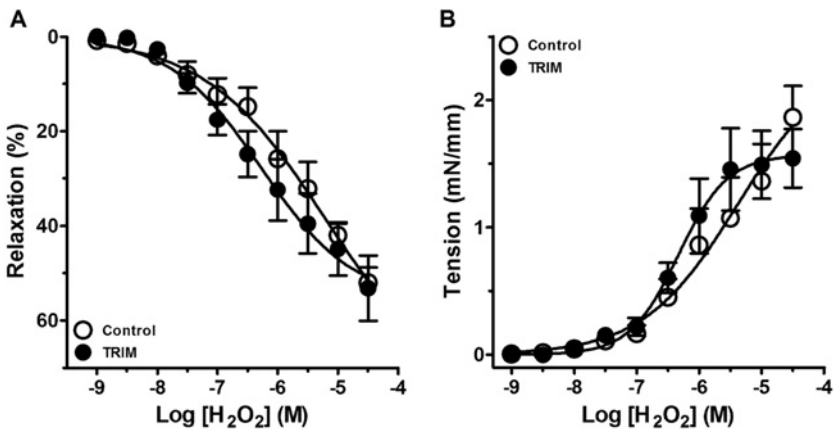


**Figure 6. Effect of hydrogen peroxide ($H_2O_2$) in endothelium-denuded (A) internal mammary artery (IMA) and (B) saphenous vein (SV) rings in the presence or in the absence of TRIM**

Results are shown as the mean ± S.E.M. A two-way ANOVA followed by Bonferroni's post-test was conducted. $n = 5$–8 for IMA and $n = 5$–6 for SV.

decreased $H_2O_2$ generation induced by ACh in the IMA. By contrast, indomethacin did not change $H_2O_2$ production compared with ACh alone. Together, these results indicate that, in the IMA, nNOS has a central role in endothelial $H_2O_2$ generation. Conversely, in SV, $H_2O_2$ production was partly inhibited, to the same extent, by L-NNA, TRIM, inhibitor-1 and indomethacin each used alone. When SV rings were treated with a combination of indomethacin + L-NNA, there was a complete inhibition of $H_2O_2$ production, indicating a role for nNOS and COX in $H_2O_2$ generation (Figure 7B).

## nNOS immunolocalization in the IMA and SV

By merging the red, green and blue components, the elastic lamellae (EL) could be safely identified in IMA sections. As observed in Figure 8A, the EL is the thickest elastic sheet, located en face to the lumen in bright cyan tonality. The characterization of EL allowed the precise distinction of nNOS immunolocalization in the endothelium and tunica media layers of the IMA (Figure 8A, red images). In sections of SV, the immunofluorescent signal for nNOS was detected on the endothelium, neointima and tunica media layers (Figure 8B).

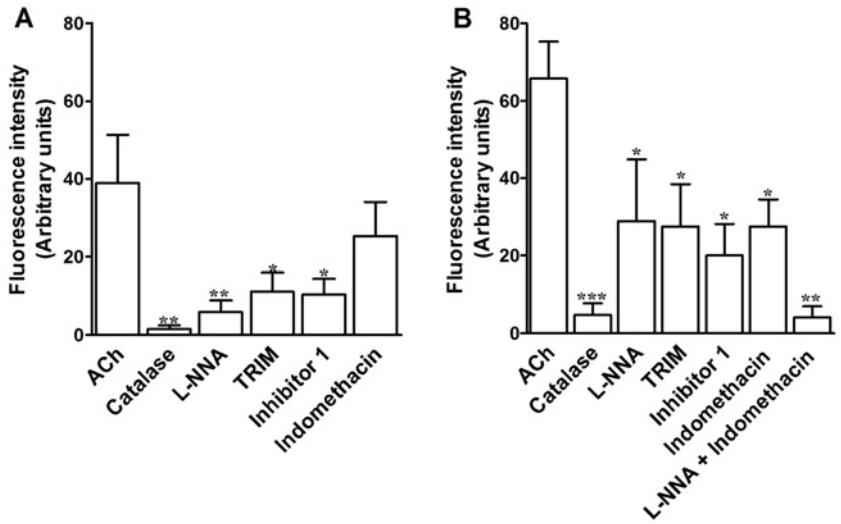


**Figure 7. Detection of acetylcholine (ACh)-induced hydrogen peroxide ($H_2O_2$) production in endothelium-intact (A) internal mammary artery ($n$ = 6–10) and (B) saphenous vein ($n$ = 6–10) in the presence or absence of catalase (2000 U/ml), L-NNA (100 $\mu$M), TRIM (300 $\mu$M), Inhibitor 1 (5 $\mu$M), Indomethacin (10 $\mu$M) and L- NNA plus Indomethacin**

Results are shown as the mean ± S.E.M. A one-way ANOVA followed by the Newman–Keuls multiple-comparison test was conducted. *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001 compared with ACh alone.

# Discussion

The major findings of this work can be summarized as follows: our experiments showed clear differences between the IMA and SV in terms of their responses to endogenous and exogenous $H_2O_2$. The most important difference was that, in the IMA, $H_2O_2$ contributed to agonist-induced vascular relaxation, whereas in the SV, agonist-stimulation of $H_2O_2$ production induced vasoconstriction. The clinical importance of this difference derives from the fact that the IMA and SV are the major conduits used as coronary artery bypass grafting.

In the USA, approximately 1,000,000 aortocoronary and peripheral vascular reconstructions are performed annually using the IMA and SV [43]. Immediate post-operative vasospasms, as well as long-term intimal hyperplasia are the leading causes of complications related to coronary arterial bypass surgery [44]. A better understanding of the physiological processes involved in the control of IMA and SV functioning should contribute to the improvement of clinical interventions regarding this common surgical procedure.

The usual assumption that endothelial NO synthase (eNOS)-derived NO is largely responsible for the regulation of vascular tone has been challenged by the discovery that a totally different molecule, $H_2O_2$, exerts an important role as a dilator of coronary and other vascular beds [9–11]. However, at least in animals, NOS is still involved in $H_2O_2$ production since this molecule is produced by nNOS along with NO in the vascular endothelium. Furthermore, $H_2O_2$ participates in agonist-induced vascular relaxation. This relatively new interaction between endogenous vasoactive mediators has been reviewed recently [45].

In this work, we investigated for the first time the role of $H_2O_2$ in the vascular reactivity of two examples of human vessels: the IMA and SV. These two vessels are particularly relevant to the coronary circulation as they are the most commonly used tissues in CABG, itself a common surgical procedure. As a result, our data could have considerable clinical significance. In agreement with previous results [46–49], we show here that the endothelium-dependent vascular relaxation in response to ACh was greater in the IMA than in the SV. In addition, in the IMA, the vasodilator response induced by ACh was completely abolished by pre-treatment with L-NAME, a non-selective NOS inhibitor; while in the SV, the vasodilator effect of ACh was only abolished by combined inhibition of COX and NOS, in line with previous reports [47,48,50]. Interestingly, pre-treatment of IMA rings with the selective nNOS inhibitors, TRIM or Inhibitor 1, significantly decreased the ACh-induced vasodilator response. Conversely, inhibition of nNOS by TRIM or Inhibitor 1, did not affect ACh-induced relaxation in SV. These results suggest that activity of nNOS contributes to vascular relaxation in the IMA, but not in the SV. These findings show, for the first time, a role for endothelial nNOS in the mechanisms underlying vascular relaxation in a human vessel. This suggestion is supported by our immunofluorescence data that show the presence of nNOS in the endothelial cells of the IMA. In agreement

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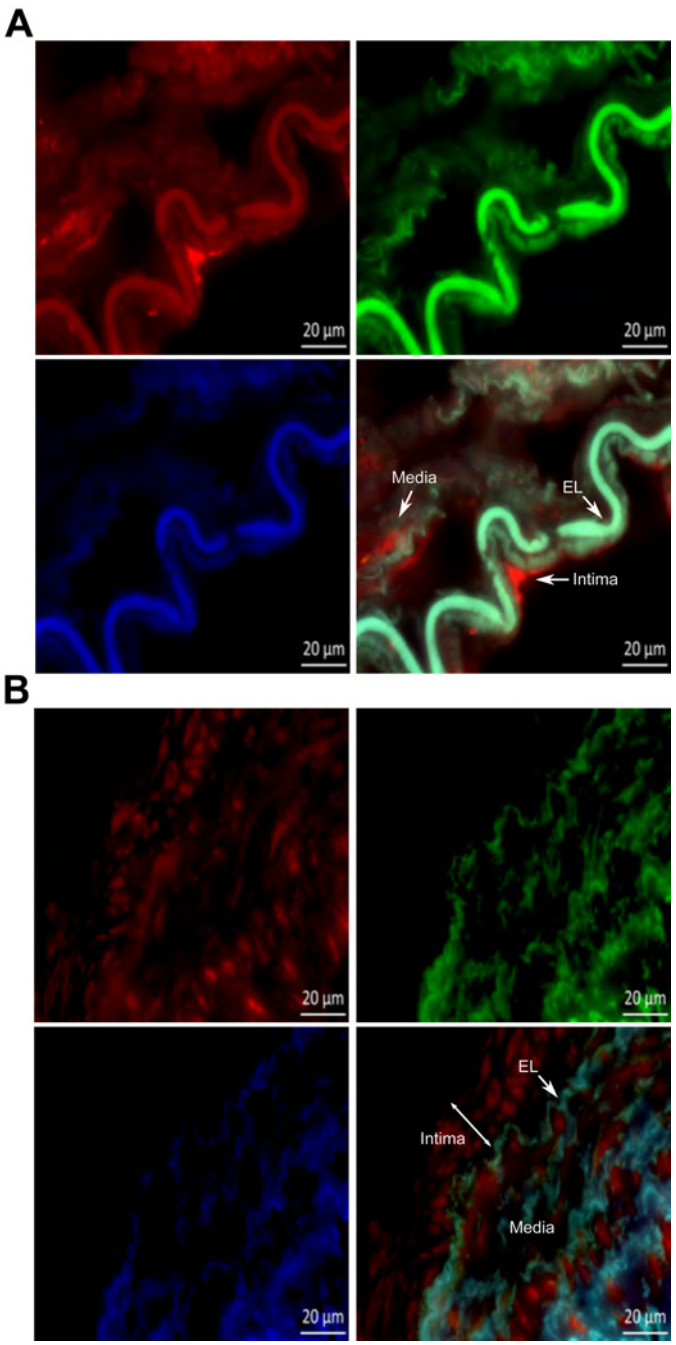


**Figure 8. Immunofluorescent staining of nNOS in human internal mammary (IMA) artery and saphenous vein (SV)**

(**A**) nNOS expression in IMA is represented by the red color image, elastin auto fluorescence in green and DAPI in blue. The superimposition of three color components facilitated the identification of the elastic lamellae (EL), seen in bright cyan tonality in merge image. In human IMA, nNOS is expressed in the endothelium and media layer (red and merge images). (**B**) In human SV, nNOS is detected in intima and media layers (red and merge images). For each vascular bed, representative images are from samples of 5 patients.

with others [51,52], our immunofluorescence data also reveal the presence of nNOS in vascular smooth muscle cells (VSMC) from human IMA and SV. However, our data suggest a major role for endothelium nNOS in mediating the vascular responses found in this work for the following reasons: (i) the effect of ACh was seen only in the presence of a functional endothelium, in agreement with previous reports [32,46]; (ii) pre-treatment of endothelium-denuded vessels with the nNOS inhibitor TRIM did not change $H_2O_2$-induced vasorelaxation in the IMA nor $H_2O_2$-induced

constriction in the SV; (iii) in the IMA, SOD decreased the vasoconstriction induced by phenylephrine only in the presence of a functional endothelium.

As highlighted above, it is known that besides NO, nNOS produces $H_2O_2$ under physiological conditions, which in some vessels behaves as an endothelium-dependent relaxant factor [21,27,28]. In the present study, we demonstrated that stimulation of IMA rings with ACh increased production of $H_2O_2$ detected fluorimetrically in the incubation mixture. This production was deeply decreased by either non-selective or selective inhibition of nNOS. As expected, the levels of $H_2O_2$ production by ACh stimulation were also markedly reduced by the addition of catalase to the rings. Inhibition of both isoforms of COX with indomethacin did not affect $H_2O_2$ production. Functionally, vascular relaxation in the rings of the IMA induced by ACh was also markedly inhibited by catalase. Finally, SOD reduced the phenylephrine-induced contraction in endothelium-containing but not in endothelium-denuded IMA rings. Together, these results suggest that stimulation of nNOS induces production of $H_2O_2$, which plays a role in the endothelium-dependent vascular relaxation of the IMA.

Curiously, our immunofluorescence data also showed the presence of nNOS in the endothelial cells of SV. Stimulation of the vessel with ACh increased the production of $H_2O_2$, which, in turn, was decreased by NOS, nNOS and COX inhibition, and by catalase. Hence, $H_2O_2$ is also produced in SV via stimulation of nNOS and COX. At this point, the isoform of COX involved in the $H_2O_2$ production in human SV remains unknown. The question that arises from these results is why nNOS and $H_2O_2$ contributed to the endothelium-dependent vascular relaxation only in the IMA. Inhibition with catalase did not change the vascular relaxation induced by ACh in the SV; however, increasing endogenous $H_2O_2$ production by SOD inhibited vascular relaxation in the SV. This last result is in line with the idea that $H_2O_2$ is inducing constriction in the SV.

Since $H_2O_2$ appeared to be an important relaxant mediator in the IMA and a vasoconstrictor in the SV, the effect of exogenous $H_2O_2$ was investigated in both vessels. Indeed, $H_2O_2$ produced a concentration-dependent relaxant effect in the IMA and a vasoconstrictor effect in the SV. Our results were in agreement with earlier studies showing that exogenous $H_2O_2$ caused a relaxation in the IMA [53] and a contraction in SV [54]. In addition, a similar opposing result was obtained with prostaglandin $E_2$ , which induced contraction in the IMA and relaxation in the SV [55]. Hence, it seems that there are important differences in the molecular mechanisms mediated by $H_2O_2$ in the control of vascular reactivity between these vessels.

Our results clearly showed a different role for $H_2O_2$ in the SV and IMA, acting as an endothelium-derived relaxant mediator in the IMA and an endothelium-derived vasoconstrictor in the SV. This difference could have important clinical implications, since $H_2O_2$ has been described as the EDHF produced by HCA in response to shear stress [10]. Thereby, $H_2O_2$ produced by the coronary bed could directly influence the vascular response in a recently implanted SV graft, leading to vasospasm, a common post-operative complication in coronary bypass surgery [4,56].

The SV remains the most used conduit in patients undergoing CABG because of the greater perioperative morbidity, mortality, duration of operation, and risk of sternal wound problems that occurs with the IMA. However, the grafted SV remains patent for a very short time due to accelerated development of atherosclerosis, as an adaptive response to the systemic circulation and surgical management [57]. Besides systemic atherogenic risk factors, the local microenvironment makes an important contribution to atherosclerotic plaque formation. Disturbance of blood flow plays an essential role in regulating local susceptibility to plaque formation through effects on endothelial cell function [58]. In this sense, vasospasm is known to affect the patency rates of CABGs [59]. So, $H_2O_2$ produced by the coronary bed may contribute to the loss of patency in the SV graft through its constrictor action on the grafted vessel. A better understanding of the particular physiological responses of the different vessels used in CABG surgery, to endogenous factors such as $H_2O_2$, could contribute to the development of novel therapeutic agents to decrease the incidence of spasm and to increase the success of this surgical procedure.

In conclusion, our data show, for the first time, a role for nNOS-derived $H_2O_2$ as an endothelium-dependent factor that has opposite effects on the smooth muscle of two types of human blood vessel. In the IMA, $H_2O_2$ acts as an endothelium-dependent relaxant factor; whereas, in SV, $H_2O_2$ has a role as an endothelium-dependent contractile factor. In view of the fact that: (i) $H_2O_2$ is the endogenous EDHF in the coronary bed; (ii) the IMA and SV are the major conduits used in CABG; and (iii) vasospasm is a common post-operative complication in coronary bypass surgery and contributes to the loss of patency of the graft, these findings may have highly relevant clinical implications.

## Clinical perspectives

- $H_2O_2$ has been identified as the EDHF in human coronary arteries and, although NO and $PGI_2$ are more important for the HCA of healthy subjects, $H_2O_2$ is gaining importance in disease states.

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nNOS is present in most vascular tissues and, in addition to NO, generates $H_2O_2$. The effects of nNOS-derived $H_2O_2$ in human vascular tissues are so far unknown.

- This study provides new information on the role of nNOS-derived $H_2O_2$ on the vascular reactivity of the human IMA and SV, the main grafts used in CABG surgery. In the IMA, $H_2O_2$, induces a relaxant effect and, in SV, $H_2O_2$ produces a contractile effect.
- Our findings provide a new insight into the pathophysiological role of $H_2O_2$ in the main grafts used in CABG surgery. Our data may also explain why complications such as vasospasms and low patency are more frequent in SV grafts than in IMA grafts.

## Author contribution

Patrick Endlich, Rosária Aires, Roberta Gonçalves, Steyner Cortes and Virginia Lemos conceived and designed the experiments. Patrick Endlich, Rosária Aires, Roberta Gonçalves, Eduardo Costa, Janaína Ângelo, Lucas Alves and Rafaela Silva performed the experiments. Patrick Endlich, Rosária Aires, Roberta Gonçalves and Lucas Alves analyzed the data. Steyner Cortes, and Virginia Lemos contributed reagents/materials/analysis tools. Patrick Endlich, Rosária Aires, Eduardo Costa, Rafaela Silva, Bruno Rezende, Steyner Cortes and Virginia Lemos wrote the manuscript.

## Competing interests

The authors declare no conflicts of interest.

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## Abbreviations

AUC, area under the curve; BSA, bovine serum albumin; CABG, coronary artery bypass graft; COX, cyclooxygenase; DCF-DA, 2′,7′ dichlorodihydrofluorescein-diacetate; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; $H_2O_2$, hydrogen peroxide; HCAs, human coronary arterioles; IMA, internal mammary artery; Inhibitor 1, (4S)-N-(4-Amino-5[aminoethyl]aminopentyl)-N′-nitroguanidine; L-NAME, $N^G$-nitro-L-arginine methyl ester; L-NNA, $N^G$-nitro-L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PECAM-1, platelet/endothelial cell adhesion molecule 1; PSS, physiological salt solution; ROS, reactive oxygen species; SV, saphenous vein; TRIM, 1-(2-trifluoromethylphenyl) imidazole; VSMC, vascular smooth muscle cells.

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