# NO Synthase but not NO, HNO or H2O2 Mediates Endothelium-Dependent Relaxation of Resistance Arteries from Patients with Resistant Cardiovascular Disease

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**Abstract**

Background and Purpose: Superoxide anions can reduce the bioavailability and actions of endothelium-derived NO. In human resistance-sized arteries, endothelium-dependent vasodilatation can be mediated by H2O2 instead of NO. We tested the hypotheses that in resistance arteries from patients with resistant cardiovascular disease (CVD), endothelium-dependent vasodilatation uses mechanisms that are either insensitive to oxidative stress or involve a reactive oxygen species. Experimental Approach: Small arteries were isolated from biopsies of the parietal pericardium of patients undergoing elective cardiothoracic surgery and were studied by immunohistochemical and organ chamber techniques. Key Results: NO-synthases 1, 2 and 3, superoxide dismutase 1 and catalase proteins were observed in the microvascular wall. Relaxing responses to bradykinin were endothelium dependent. During submaximal depolarization-induced contraction, these relaxations were inhibited by inhibitors of NO-synthases (NOS) and soluble guanylyl cyclase (sGC) but not by scavengers of NO or HNO, inhibitors of cyclooxygenases, neuronal NO-synthase, superoxide dismutase or catalase, or by exogenous catalase. During contraction stimulated by endothelin-1, these relaxations were not reduced by any of these interventions except DETCA which caused a small reduction. Conclusion and Implications: In resistance arteries from patients with resistant CVD, endothelium-dependent relaxations seem not to be mediated by NO, HNO or H2O2 although NOS and sGC can be involved. These vasodilator responses proceed during excessive oxidative stress.

**NO Synthase but not NO, HNO or $H_2O_2$ Mediates Endothelium-Dependent Relaxation of Resistance Arteries from Patients with Resistant Cardiovascular Disease.**

**Running title:** Endothelium in patients' resistance arteries

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**Abstract**

**Background and Purpose:** Superoxide anions can reduce the bioavailability and actions of endothelium-derived NO. In human resistance-sized arteries, endothelium-dependent vasodilatation can be mediated by $H_2O_2$ instead of NO. We tested the hypotheses that in resistance arteries from patients with resistant cardiovascular disease (CVD), endothelium-dependent vasodilatation uses mechanisms that are either insensitive to oxidative stress or involve a reactive oxygen species.

**Experimental Approach:** Small arteries were isolated from biopsies of the parietal pericardium of patients undergoing elective cardiothoracic surgery and were studied by immunohistochemical and organ chamber techniques.

**Key Results:** NO-synthases 1, 2 and 3, superoxide dismutase 1 and catalase proteins were observed in the microvascular wall. Relaxing responses to bradykinin were endothelium dependent. During submaximal depolarization-induced contraction, these relaxations were inhibited by inhibitors of NO-synthases (NOS) and soluble guanylyl cyclase (sGC) but not by scavengers of NO or HNO, inhibitors of cyclooxygenases, neuronal NO-synthase, superoxide dismutase or catalase, or by exogenous catalase. During contraction stimulated by endothelin-1, these relaxations were not reduced by any of these interventions except DETCA which caused a small reduction.

**Conclusion and Implications:** In resistance arteries from patients with resistant CVD, endothelium-dependent relaxations seem not to be mediated by NO, HNO or $H_2O_2$although NOS and sGC can be involved. These vasodilator responses proceed during excessive oxidative stress.

**Keywords:** Bradykinin, coronary artery disease, endothelin-1, oxidative stress, superoxide dismutase

**Abbreviations**

7-NI 7-nitroindazole

BK Bradykinin

CCRC Cumulative concentration response curve

c-PTIO 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

CVD Cardiovascular diseases

CXL-1020 N-hydroxy-2-(methylsulfonyl)benzenesulfonamide

DETCA 3,17-diacetoxyestra-1,3,5(10)-trien-2-carboxylic acid

ET-1 Endothelin-1

L-NAME $N^{\omega}$-nitro-L-arginine methyl ester

NAC N-acetyl cysteine

NPLA $N^{\omega}$-propyl-L-arginine

ODQ 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

PSS Physiological salt solution

ROS Reactive Oxygen Species

SNP Sodium-nitroprusside

## 1 Introduction

NO produced by endothelial cells and stimulating production of cGMP in the underlying smooth muscle cells, is repeatedly proposed to mediate endothelium-dependent vasodilatation(Monica, Bian & Murad, 2016; Shimokawa & Godo, 2020; Vanhoutte, Shimokawa, Feletou & Tang, 2017; Vanhoutte, Zhao, Xu & Leung, 2016). Superoxide anions ($O_2^-$) which rapidly bind and inactivate NO, can then cause endothelial dysfunction during conditions of increased oxidative stress that take part in the pathogenesis of cardiovascular diseases (CVD)(Daiber et al., 2017; Forstermann & Munzel, 2006; Zhang, Murugesan, Huang & Cai, 2020). Reported experimental evidence for these widely accepted mechanisms of vasodilatation is frequently incomplete and does not exclude alternative mechanisms. In addition to de novo synthesis, endothelial NO can be released from intracellular stores and can relax vascular smooth muscle independently from cGMP(Batenburg, Garrelds, van Kats, Saxena & Danser, 2004; Batenburg et al., 2004). NO-synthases (especially the nNOS isoform) produce not only NO but also nitroxyl(Fukuto, 2019; Irvine, Ritchie, Favaloro, Andrews, Widdop & Kemp-Harper, 2008; Schmidt, Hofmann, Schindler, Shutenko, Cunningham & Feelisch, 1996; Toda & Okamura, 2003). Like NO, HNO is a potent stimulator of soluble guanylyl cyclase (sGC) but resists inactivation by $O_2^-$(Arcaro, Lembo & Tocchetti, 2014; Irvine, Ritchie, Favaloro, Andrews, Widdop & Kemp-Harper, 2008; Leo, Joshi, Hart & Woodman, 2012). In small muscular resistance-sized arteries, the relation between oxidative stress in CVD and endothelium-dependent vasodilatation is even more complex than in large elastic conduit arteries(Ellinsworth, Sandow, Shukla, Liu, Jeremy & Gutterman, 2016; Shimokawa & Morikawa, 2005; Vanhoutte, Shimokawa, Feletou & Tang, 2017). These micro-arteries which control blood pressure and local blood flow, continue to display endothelium-dependent vasodilatation when NOS or sGC are inhibited. In human resistance arteries, this additional pathway may involve $H_2O_2$ produced by the microvascular endothelial cells and causing hyperpolarization of the underlying smooth muscle(Ellinsworth,

Sandow, Shukla, Liu, Jeremy & Gutterman, 2016; Leurgans et al., 2016; Shimokawa & Morikawa, 2005; Vanhoutte, Shimokawa, Feletou & Tang, 2017). Dismutation of $O_2^-$ produced by mitochondria, NOS or NADPH oxidases is the main source of this endothelium-derived hyperpolarizing $H_2O_2$(Shimokawa & Godo, 2020; Widlansky & Gutterman, 2011; Zhang, Murugesan, Huang & Cai, 2020). In human resistance arteries, increased oxidative stress can thus blunt actions of endothelium-derived NO and promote endothelium-dependent hyperpolarization. The situation is unclear in resistance arteries from patients with resistant CVD requiring surgery. Here, chronic elevation of oxidative stress resulting from multiple risk factors and a long history of CVD might impair the NOS/NO/sGC pathway and promote endothelium-dependent hyperpolarization while chronic treatment with cholesterol lowering, anti-hypertensive and anti-diabetic drugs that have direct and indirect antioxidant properties(Daiber et al., 2017), can protect endothelium-derived NO. Recent observations by Gutterman et al. on endothelium-dependent flow-induced dilatations of human resistance arteries are partly in line with this proposal. In contrast to vessels from patients without clinical signs of coronary artery disease, these dilatations were not inhibited by the non-specific inhibitor of NO-synthases L-NAME but were blocked by scavenging of $H_2O_2$ with catalase in vessels isolated from patients undergoing coronary artery bypass grafting(Freed, Beyer, LoGiudice, Hockenberry & Gutterman, 2014; Schulz, Katunaric, Hockenberry, Gutterman & Freed, 2019; Zinkevich, Fancher, Gutterman & Phillips, 2017).

In this study, we tested the hypotheses that endothelium-dependent vasodilatation of resistance arteries from patients with resistant CVD uses mechanisms that are either insensitive to oxidative stress or involve a reactive oxygen species. For this purpose, we isolated resistance arteries from biopsies of parietal pericardium obtained during elective cardiothoracic surgeries. In view of the small size of the arterial tissue samples, we used immunohistochemistry and pharmacological tools to demonstrate presence of mechanistic components and their contribution to the in vitro relaxing effects of the endothelium-dependent vasodilator bradykinin.

## 2 Material and Methods

### *2.1 Patients and ethics*

From 55 patients, a biopsy of the parietal pericardium (2x2 cm) was taken at the initiation of elective cardiothoracic surgery (coronary artery bypass grafting and/or cardiac valve replacement surgery) and stored in sterile physiological salt solution (PSS) at 4 °C as previously described(Leurgans et al., 2016). Informed written consent was obtained from all patients prior to surgery. The study was approved by the Regional Committees of Health Research Ethics for Southern Denmark (S-20140202) and experiments were conducted according to the principles expressed in the Declaration of Helsinki of the World Medical Association(World Medical, 2013). Characteristics of the patients are summarized in Table 1.

### *2.2 Recording of vasomotor responses*

The biopsies were stored for 16 h at 4 °C in 30 mL PSS to wash away anesthetics and analgesics(Batenburg, Garrelds, van Kats, Saxena & Danser, 2004). They were then transferred to PSS at room temperature (RT, 220C) and one long arterial resistance segment (6-12 mm) was isolated from the pericardial sheet, cleaned from connective tissue, and divided into 2 mm segments. These preparations were mounted in multi-chamber wire myographs (DMT 620M, Danish Myo Technology, Aarhus, Denmark) to record isometric tension development. The organ chambers contained 5 mL PSS maintained at 37 °C and continuously aerated with 5% $CO_2$ in air. Prior to pharmacological analysis, segments were stretched to a lumen diameter and resting wall tension corresponding to a transmural pressure of 100 mmHg according to the law of Laplace. We previously observed that under these conditions this type of arterial preparation developed the strongest contractile responses (Leurgans et al., 2016). After 5-7 hours of pharmacological study, the distended arterial segments were fixed by overnight incubation in 4% formaldehyde solution at RT and then stored in PBS/0.05% sodium-azide at 40C for subsequent immunohistochemical analyses.

### *2.3 Pharmacological protocols*

We compared effects of inhibitors of nitrosative and oxidative mechanisms (see Table 2) on arterial relaxing

responses to i) reference agents and to ii) the endothelium-dependent vasodilator bradykinin (BK), during depolarization- and agonist-induced submaximal contraction. The isolated resistance artery segments were first exposed 3 times during 5 min at 20 min interval to 32 mM $K^+$. We previously showed that this stimulus causes a contraction that is [?]40 % of the maximal response to a contractile agonist such as ET-1(Leurgans et al., 2016). In 2 out of 55 experiments, 32 mM $K^+$ resulted in a contraction that was smaller than 0.2 $N.m^{-1}$ and these experiments were discontinued. During the $3^{rd}K^+$-induced contraction, the arterial segments were exposed to 1 µM BK for 3 min Following these initial tests, the preparations were incubated for 20 min without (control) or with a pharmacological inhibitor (treated) and relaxing responses to a NO-donor, a nitroxyl-donor compound or exogenous $H_2O_2$ were investigated during contraction stimulated with 32 mM $K^+$. Two to five different treatments were always investigated in parallel with one control arterial segment from the same individual patient. After washout of the inhibitor and of the contractile and relaxing stimuli, similar experiments were performed in series evaluating effects of the pharmacological treatments on relaxing responses to BK during contraction stimulated with 32 mM $K^+$. Ultimately, after at least 20 min recovery from this contraction/relaxation cycle, effects of the same pharmacological treatments were tested on relaxing responses to BK during contraction stimulated with ET-1. The concentration of ET-1 was progressively titrated (from 0.063 nM) so that the amplitude of the agonist-induced contraction would match that of the contraction stimulated by 32 mM $K^+$ in the same arterial preparation. In addition to a fair comparison between depolarizing and contractile agonist-induced conditions, this approach allowed to evaluate the effects of the pharmacological inhibitors on the sensitivity of the resistance arteries to ET-1. In initial experiments that evaluated effects of L-NAME, c-PTIO and ODQ, we focused on the maximal response elicited by 1 µM BK. Because an unexpected (lack of) effect was observed, cumulative concentration response curves with half logarithmic steps were constructed for BK (0.01 nM to 1.0 µM) in subsequent experimental series.

### *2.4 Immunohistochemistry*

Presence and distribution of NOS, SOD1 and catalase were determined in arteries from a subset of 28 randomly selected patients. Four µm thick cross-sections of the formalin-fixed paraffin embedded arterial segments were obtained and mounted on SuperFrost Plus Adhesion slides (Thermo Fisher Scientific, Waltham, MA, USA). Samples were fixed on the glass slides at 60°C for 30 min and then deparaffinized and rehydrated through a series of Tissue-Clear (Sakura, Alphen aan den Rijn, The Netherlands), and graded ethanol (99-70%, Merck). Antigens were retrieved by heating in sodium citrate buffer (10 mM, pH6, Sigma-Aldrich) at 970C in an oven for 22 min. and then cooled on ice for 25 min. Blocking of endogenous peroxidase was performed in 0.3% $H_2O_2$and 50 mM $NH_4Cl$ in PBS for 10 min at RT. Blocking was performed using PBS/0.025% Triton for 30 min. Primary antibodies were diluted and applied in the blocking buffer as follows; CAT (1:1000, ab52477, Abcam, Cambridge, UK), eNOS (1:100, PA1-037, Invitrogen$^{TM}$ via Fischer Scientific, Roskilde, Denmark), iNOS (1:300, PA1-036, Fischer Sci), nNOS (1:400, ab1376, Abcam) and SOD1 (1:400, MA1-105, Invitrogen$^{TM}$ via Fischer Sci ) 30 min at RT and then overnight at 4°C. Secondary peroxidase-conjugated antibodies (Dako, Glostrup, Denmark,goat-anti-mouse (for SOD1), goat-anti-rabbit (for eNOS, CAT), mouse-anti-goat (for nNOS)) were diluted 1:200 and applied in blocking buffer for 1 hour at RT. Staining was visualized with 3,3'-diaminobenzidine (DAB, Dako), sections were counterstained for 2 min with Mayer's Hematoxylin (Sigma-Aldrich), and imaged using an Olympus BX51 microscope equipped with a DP26 camera and CellSens software (Olympus). Presence of immunohistochemical staining was semi-quantitively analyzed by rating the stainings (0 = no staining; 4 = strong staining). Rating was performed by two independent observers that were blinded to the sections' IDs. Sections exposed to the secondary but not the primary antibody were used as negative controls. Sections of human kidney, thoracic aortic aneurysm, atrial appendage, or parietal pericardium, formalin-fixed directly after isolation during surgery, served as positive controls.

### *2.5 Statistics*

The data and statistical analyses comply with the recommendations of the*British Journal of Pharmacology* on experimental design and analysis in pharmacology(Curtis et al., 2018). Data were analyzed using GraphPad

Prism (version9, GraphPad Software, San Diego, CA, USA) and summary data are shown as mean ± SEM for small data sets (N < 20, under the assumption of normal distribution) and as median and interquartile range for larger data sets (N > 30, not meeting normal distribution). Data on the effect of pharmacological tools on the contractile responses to $K^+$ are shown as the percentage of the second contraction induced by 32 mM $K^+$. Data on the effect of inhibitors on contractions induced by ET-1 are shown as the concentration of ET-1 required to achieve amplitude-matching to contractions induced by 32 mM $K^+$. Relaxations were calculated as the percentage reduction of the contraction. Individual cumulative-concentration response curves (CCRCs) were fitted using a non-linear regression curve with variable slope. Effects of inhibitors were studied in parallel with an untreated control and statistical analyses were carried out under the assumption of normal distribution by comparisons of the potency ($pD_2$ values) and efficacy ($E_{max}$ values) using one-way mixed-effects model ANOVA. A Dunnett's post-hoc test was used to correct for multiple comparisons. Results of experiments in arterial segments from the same patient and in those from different patients, were compared by paired two-sided $t$ -test and by unpaired two-sided $t$ -test, respectively. $P < 0.05$ was accepted to denote statistical significance

*2.6 Materials*

Physiological salt solution (PSS) contained (in mM) NaCl (115), $NaHCO_3$ (25), $K_2HPO_4$(2.5), $MgSO_4$ (1.2), Glucose (5.5), HEPES (10) and $CaCl_2$ (1.3). For PSS containing 32 instead of 5 mM $K^+$, the NaCl concentration was reduced to 88 mM and 27 mM KCl was added. Buffers where continuously aerated with 5% $CO_2$ in air at 37 °C. Bradykinin (BK; Sigma-Aldrich) was dissolved in 0.1 M acetic acid. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (c-PTIO; Sigma-Aldrich) and 7-nitroindazole (7-NI; Sigma-Aldrich), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Cayman Chemical, Ann Arbor, Mi, USA) and N-hydroxy-2-(methylsulfonyl)benzenesulfonamide (CXL-1020; Axon Medchem, Groningen, NL) were dissolved in DMSO. Indomethacin (INDO; Sigma-Aldrich) and N-omega-propyl-L-arginine (NPLA; Sigma-Aldrich) were dissolved in 100% EtOH. CXL-1020 (Axon Medchem, Groningen, The Netherlands), endothelin-1 (ET-1; Bachem, Weil am Rhein, Germany), hydrogen peroxide ($H_2O_2$; Sigma-Aldrich), N-omega-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) and sodium nitroprusside (SNP; Sigma-Aldrich) were dissolved in dd$H_2O$. 3-Amino-1,2,4-triazole (Amitrole; Sigma-Aldrich), catalase (CAT; Sigma-Aldrich), 3,17-diacetoxyestra-1,3,5(10)-trien-2-carboxylic acid (DETCA; Sigma-Aldrich) and N-acetylcysteine (NAC; Sigma-Aldrich) were dissolved in PSS. The biological effects and concentrations of the pharmacological tools that we used are summarized in Table 2.

*2.7 Nomenclature of targets and ligands*

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY*http://www.guidetopharmacology.org*and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019a; Alexander et al., 2019b).

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(Alexander et al., 2019).

## 3 Results

*3.1 General properties of patients*

Table 1 summarizes properties of the patients (N = 53). Males (62 %) and females (38 %) were undergoing elective cardiothoracic surgery for coronary artery bypass grafting, replacement of cardiac valves or both. Their cardiovascular disease had progressed despite treatment with anti-coagulant drugs, blood pressure-, cholesterol- and/or blood glucose lowering drugs and (on average) normalization of risk factors such as

hypertension, hyperlipidemia and hyperglycemia. They thus represent patients with resistant cardiovascular disease.

*3.2 General properties of resistance arteries*

The lumen diameter of isolated and distended pericardial small arteries averaged 243 ± 19 µm (mean of means; n=225 segments, N=53 patients). The contractile response of these preparations to depolarization with 32 mM $K^+$ was 1.47 ± 0.40 $N.m^{-1}$ and exhibited a weak statistically significant positive relationship to the lumen diameter of the vessels (Figure 1A, 1B and 1D). In 3/53 experiments, 1 µM BK failed to induce a noticeable arterial relaxation. These arterial preparations were not investigated further. In the remaining experiments, the relaxing response to 1 µM BK averaged -65 ± 12% (mean of means; n = 205, N = 50). It differed considerably between patients (Figure 1C) but was not significantly related to the absolute amplitude ($N.m^{-1}$) of the pre-contraction. It was significantly (but not markedly) smaller for patients with coronary artery disease (-57 ± 4%; n = 86, N = 21) compared to those needing replacement of cardiac valves (-71 ± 5%; n = 91, N = 22, $P = 0.041$). In a small number of dedicated experiments, gentle mechanical damage to the luminal surface of the arterial segments abolished the relaxing response to 1 µM BK during contraction induced by 32 mM $K^+$ (Figure 2).

In arteries that contracted in response to depolarizing solution, contraction was also stimulated by ET-1. This agonist can increase oxidative stress(Davenport et al., 2016) and has been proposed to shift the mediator of endothelium-dependent relaxation from NO to $H_2O_2$(Leurgans et al., 2016). On average, 3.36 ± 0.72 nM ET-1 resulted in contractions that were comparable to those stimulated by 32 mM $K^+$ in the same arterial segments (2.10 ± 0.19 $N.m^{-1}$ vs.1.52 ± 0.17 $N.m^{-1}$, respectively, n = 36, N = 36). In the presence of ET-1, relaxations induced by 1 µM BK were significantly larger than during $K^+$-stimulated contraction (-78 ± 2% vs. -68 ± 3% respectively, N = 36, $P = 0.034$) and were also endothelium-dependent (Figure 2).

*3.3 Presence of NO-synthases and antioxidant enzymes*

Endothelial NO-synthase (eNOS or NOS3) was detected with immunohistochemistry in most arteries investigated (Figure 3A). The staining was faint, unevenly distributed over the arterial wall, not restricted to the luminal endothelial layer but present in the tunica media as well. Largely similar staining for eNOS was observed in small arteries in the atrial appendage fixed immediately after isolation during surgery (Figure 3B). This control was performed to exclude that expression of eNOS had been modified during several hours of in vitro investigation of the isolated resistance arteries investigated.

The other isozymes of NO-synthase were expressed as well in the resistance arteries of the CVD patients. As was the case for eNOS, staining of inducible NO-synthase (iNOS or NOS2) was faint, unevenly distributed over the microvascular wall and detected in both endothelial and smooth muscle cells (Figure 3C). Even though not directly comparable due to the nature of the IHC, expression of neuronal NO-synthase (nNOS or NOS1) was easily detectable, and thus seemed more abundant in the resistance artery endothelium and smooth muscle compared to the other NO-synthases and was often detectable in the tunica adventitia as well (Figure 3D).

Endogenous catalase and Cu-Zn superoxide dismutase (SOD1) were detected in far less patients compared to the NO-synthases and both were observed primarily in the arterial smooth muscle cells (Figure 3E and 3F).

*3.4 Relaxing responses to exogenous NO, HNO and $H_2O_2$*

One µM Na-nitroprusside (SNP, NO-donor), reduced contractile responses to 32 mM $K^+$ (-58 ± 6%, N = 9; Figure 4A). L-NAME tended to increase this relaxing effect (-74 ± 5%, N = 9) but this did not reach statistical significance ($P = 0.054$). c-PTIO and ODQ abolished relaxing responses to 1 µM SNP (Figure 4A). Indomethacin did not modify the relaxing effect of SNP and its inhibition by c-PTIO and ODQ (Figure 4B). Relaxing responses to 0.001 – 1 µM SNP during $K^+$-induced contraction were not modified by NAC which scavenges HNO but not NO (Figure 4C). DETCA had a profound inhibitory effect on relaxing responses to SNP while amitrole slightly increased its efficacy (Figure 4C).

CXL-1020 was less potent than SNP but at least as efficacious in relaxing depolarized human pericardial resistance arteries (Figure 4D). The potency of this HNO-donor compound was significantly reduced in the presence of NAC which did not reduce relaxing responses to SNP. DETCA and amitrole, on the other hand, did not modify the relaxing effects of CXL-1020 (Figure 4D).

During contraction stimulated with 32 mM $K^+$, exogenous $H_2O_2$caused relaxations with smaller potency but similar efficacy, compared to SNP and CXL-1020 (Figure 5A). $H_2O_2$was approximately 10 times more potent in reducing contractions induced by ET-1 (Figure 5B). Irrespective of the type of contractile stimulus, relaxing effects of $H_2O_2$ were not significantly modified by L-NAME but, even at high concentrations, abolished in the presence of exogenous catalase (Figure 5).

*3.5 Basal oxidative and nitrosative stress*

The inhibitors of cyclooxygenases, NO-synthases and antioxidant enzymes and the scavengers of reactive oxygen species that we used, did not cause contraction in the isolated pericardial resistance arteries. L-NAME significantly increased contractile responses to 32 mM $K^+$ by 48 ± 11 % ($P < 0.0001$; N = 28) (Figure 6). It did not increase the potency of ET-1 further; 3.1 ± 0.8 and 2.4 ± 0.6 nM ET-1 were needed to cause contractions similar to those stimulated by 32 mM $K^+$ in the presence and absence of L-NAME, respectively ($P = 0.1754$; N = 27) (Suppl. Figure 1). In contrast to the non-selective NOS-inhibitor L-NAME, the selective inhibitors of nNOS 7-NI and NPLA did not increase contractile responses to 32 mM $K^+$ (Figure 6). Also, c-PTIO and DETCA, which abolished relaxing responses to SNP (Figure 4), did not increase contractile responses to 32 mM $K^+$ (Figure 6). Although exogenous catalase did not significantly modify contractile responses, amitrole (a putative inhibitor of catalase) reduced $K^+$-induced contractions by -48 ± 9 % ($P < 0.001$; N = 11).

*3.6 Endothelium-dependent relaxations*

During contraction stimulated by 32 mM $K^+$, the relaxing response to 1 μM BK was significantly reduced by L-NAME and by ODQ (Figure 7A and 7B). On average, the effect of both inhibitors was only partial and was more marked in the presence of indomethacin. Surprisingly, in both the absence and presence of indomethacin, c-PTIO did not alter relaxing responses to BK in depolarized arteries in which it abolished relaxing responses to SNP (Figure 7A and 7B, Figure 4A and 4B). Also, in contrast to exogenous NO, DETCA partly reduced relaxing responses to 0.01 nM – 1 μM BK but did not abolish them (Figure 7C). We therefore evaluated involvement of HNO which can be generated most notably by nNOS which in addition to eNOS is also expressed in the wall of the pericardial resistance arteries (Figure 3). However, neither the HNO-scavenger NAC nor the selective nNOS-inhibitors 7-NI and NPLA modified BK-induced relaxation in arteries in which L-NAME caused a partial inhibition (Figure 7C and Figure 8A). While amitrole moderately but significantly increased the potency and efficacy of the relaxing effect of BK during $K^+$-induced contraction (Figure 7C), exogenous catalase did not modify them in the absence and presence of L-NAME (Figure 7D).

Effects of the various inhibitors were not only tested on BK-induced relaxation during contractions stimulated by 32 mM $K^+$but also on BK-induced relaxation during contractions of similar amplitude stimulated by ET-1 in the same arterial segments (Figures 8 and 9). Indomethacin did not modify responses to BK during agonist-stimulated contraction (Figure 9B). In most experiments, also L-NAME failed to modify these responses to BK (Figure 8B, 9A, 9B), but in one experiment a small statistically significant reduction by L-NAME was observed (Figure 9D). In line with the on average failure of L-NAME to blunt BK-induced relaxation of ET-1-induced contraction, also 7-NI, NPLA, c-PTIO, NAC and ODQ were without effect (Figure 8B, 9A, 9B and 9C). While DETCA slightly but significantly reduced responses to BK, amitrole and exogenous catalase did not modify BK-induced relaxation during ET-1 stimulated contraction in both the absence and presence of L-NAME (Figure 9C and 9D). This contrasted with the effects of these inhibitors on the responses of the same arterial preparations to exogenous NO and $H_2O_2$ (Figure 4C and Figure 5B).

On average, he inhibitory effect of L-NAME on the maximal relaxing response of depolarized arteries to BK (from -63 ± 6 to -23 ± 4% and from -68 ± 5 to -30 ± 7%) and its effect on the relaxation of arteries precontracted with ET-1 (from -76 ± 8 to -61 ± 8% and from -76 ± 5 to -68 ± 9%) did not differ significantly

between patients with coronary artery disease (N = 11) and patients that did not require bypass surgery (N = 9) (Figure 10). In both groups of patients, not only the BK-induced relaxation but also the extent to which it could be reduced by L-NAME differed considerably between individuals (Figure 10).

## 4 Discussion

The main findings of this study are that BK-induced relaxations of resistance arteries from patients with resistant CVD, are not modified by c-PTIO, NAC or catalase and only slightly reduced by DETCA. They suggest that extracellular NO, HNO and $H_2O_2$ do not mediate communication from endothelium to smooth muscle during endothelium-dependent relaxation of resistance arteries from these patients and that these relaxations can proceed during excessive oxidative stress.

Endothelium-dependent vasodilatation of human resistance arteries are mediated by endothelium-derived prostacyclin, NOS and endothelium-dependent hyperpolarization in children, adults and patients with coronary artery disease, respectively(Beyer et al., 2017). Several laboratories including our own, reported that $H_2O_2$ can act as an endothelium-derived hyperpolarizing factor in resistance arteries from patients(Beyer et al., 2017; Ellinsworth, Sandow, Shukla, Liu, Jeremy & Gutterman, 2016; Leurgans et al., 2016; Schulz, Katunaric, Hockenberry, Gutterman & Freed, 2019; Shimokawa & Godo, 2020; Shimokawa & Morikawa, 2005). Relations between this factor, NOS and oxidative stress are controversial and were the focus of our study. The functional importance of endothelium-dependent hyperpolarization is largely unknown because it is usually investigated during pharmacological inhibition of NO-synthases. In large elastic conduit arteries, endothelial NOS produces NO that causes smooth muscle relaxation via production of cGMP. Superoxide anions produced by NADPH oxidases and mitochondria inhibit this pathway by uncoupling of eNOS, binding and inactivation of NO and damaging sGC(Daiber et al., 2017; Elbatreek et al., 2020; Evgenov, Pacher, Schmidt, Hasko, Schmidt & Stasch, 2006; Forstermann & Munzel, 2006; Monica, Bian & Murad, 2016; Zhang, Murugesan, Huang & Cai, 2020). In addition to NO, NOS can produce HNO and $O_2^-$(Forstermann & Munzel, 2006; Schmidt, Hofmann, Schindler, Shutenko, Cunningham & Feelisch, 1996). Nitroxyl is not inactivated by $O_2^-$ and potently stimulates sGC(Fukuto, 2019; Irvine, Ritchie, Favaloro, Andrews, Widdop & Kemp-Harper, 2008; Leo, Joshi, Hart & Woodman, 2012; Toda & Okamura, 2003). Although L-NAME partly inhibits production of $O_2^-$ by endothelial NOS(Kaesemeyer, Ogonowski, Jin, Caldwell & Caldwell, 2000), superoxide synthesized by NOS is transformed by SOD1 into $H_2O_2$ as an endothelium-derived hyperpolarizing factor in mouse mesenteric resistance arteries(Morikawa et al., 2003; Takaki et al., 2008). In coronary arterioles from patients with coronary artery disease, on the other hand, mitochondria rather than NOS are the source of vasodilator endothelium-derived $H_2O_2$(Beyer et al., 2017; Freed, Beyer, LoGiudice, Hockenberry & Gutterman, 2014; Schulz, Katunaric, Hockenberry, Gutterman & Freed, 2019).

With the aim to analyze clinically relevant relations between endothelium-dependent vasodilatation and oxidative mechanisms, we tested the hypotheses that BK-induced relaxation of resistance arteries from patients with resistant CVD is mediated by mechanisms that are either insensitive to oxidative stress or involve a reactive oxygen species. For this purpose, we studied resistance artery biopsies obtained during elective cardiothoracic surgery. The demographic, clinical and vascular pharmacological properties of the study group varied (Table 1 and Figure 1). Surgery was required despite normalization of classical risk factors, suggesting future benefit from novel pharmacotherapy inspired by proven pathogenic mechanisms. Resistance artery contractile and relaxing responses varied considerably between individual patients (Figure 1). The different size of the vessels, which is influenced by sampling bias, contributed little to this inter-assay variability. Because some of the results were unexpected, we had to confirm the endothelium-dependence of BK-induced relaxing responses in patient pericardial resistance arteries(Leurgans et al., 2016). Immunohistochemical staining of potential key players also displayed considerable inter-individual variability despite careful titration of the concentration of the primary antibodies. But, presence of all three isoforms of NOS, SOD1 and catalase could be demonstrated in the microvascular wall.

To probe involvement of mediators in relaxing responses we tested effects of exogenously applied candidate mediators and of potential scavengers thereof. We confirmed that SNP, a NO-donor, and exogenous $H_2O_2$cause relaxation in patient pericardial resistance arteries(Leurgans et al., 2016; Leurgans, Bloksgaard,

Irmukhamedov, Riber & De Mey, 2018). We found that CXL-1020, an HNO-donor compound(Arcaro, Lembo & Tocchetti, 2014), relaxes patient resistance arteries. Exogenous catalase prevented relaxing effects of exogenous $H_2O_2$, as expected. c-PTIO, ODQ and DETCA abolished relaxing effects of SNP confirming that they are due to NO stimulating sGC and sensitive to inactivation by $O_2^-$(Forstermann & Munzel, 2006; Ignarro et al., 1980; Omar, Cherry, Mortelliti, Burke-Wolin & Wolin, 1991; Vanhoutte, Zhao, Xu & Leung, 2016). NAC did not modify responses to SNP but reduced the relaxing potency of CXL-1020 which was not altered by the SOD-inhibitor DETCA. These properties are very similar to those of nitrergic nervous dilator mechanisms in various organs that are mediated by HNO generated by neuronal NOS, insensitive to scavenging and inactivation by endogenous $O_2^-$ or c-PTIO but sensitive to inhibition by NAC(Arcaro, Lembo & Tocchetti, 2014; Fukuto, 2019; Irvine, Ritchie, Favaloro, Andrews, Widdop & Kemp-Harper, 2008; Schmidt, Hofmann, Schindler, Shutenko, Cunningham & Feelisch, 1996; Toda & Okamura, 2003). Apart from exogenous catalase, the actions of the pharmacological tools that we revalidated are not restricted to the extracellular space.

DETCA abolished relaxing responses to exogenous NO during $K^+$-induced contraction and amitrole reduced these contractions. This suggests a large production of endogenous $O_2^-$leading via dismutation to $H_2O_2$ that is then decomposed by endogenous catalase. This endogenous antioxidant pathway seems efficacious because addition of exogenous catalase did not modify contractile responses. No signs of basal production of relaxing concentrations of HNO were observed since NAC, 7-NI and NPLA did not modify $K^+$-induced contractions. In contrast to the selective inhibitors of nNOS, L-NAME increased contractile responses. This finding has frequently been interpreted as indicative of basal production of NO. However, c-PTIO and DETCA did not increase contractile responses and the effect of ODQ did not reach statistical significance (Figure 6A). Non-canonical effects of L-NAME that are not due to inhibition of NOS activity have been observed after chronic but not acute administration of the compound(Kopincova, Puzserova & Bernatova, 2012; Liu et al., 2019). Our findings therefore raise the possibility that, at least in resistance arteries from patients with CVD, NOS can generate a dilator compound that is distinct from NO, HNO and $O_2^-$.

We studied agonist-stimulated endothelium-dependent relaxation during i) contraction stimulated by $K^+$-induced depolarization that mimics myogenic tone and inhibits hyperpolarizing influences and during ii) contraction stimulated with ET-1. This stimulator of NADPH oxidases is upregulated in several CVD (Barton & Yanagisawa, 2019; Davenport et al., 2016). In patient pericardial resistance arteries, its vasoconstrictor effect is mediated by $ET_A$ receptors and not modified by direct or endothelium-dependent $ET_B$effects(Leurgans et al., 2016). We confirmed that in these vessels, BK-induced relaxation of ET-1 stimulated contraction is larger and refractory to inhibition by L-NAME and ODQ compared to $K^+$-induced contraction(Leurgans et al., 2016). Our earlier proposals on involvement of endothelium-derived NO during depolarization-induced contraction and of endogenous $H_2O_2$ as an endothelium-derived hyperpolarizing factor in the presence of ET-1, could however not be confirmed.

In depolarized arteries, the endothelium-dependent effects of BK were on average markedly reduced by L-NAME and ODQ. Unlike those of SNP, they were not modified by c-PTIO and only partly reduced by DETCA. These properties could suggest involvement of HNO instead of NO. Although the nitroxyl donor CXL-1020 caused relaxation with an efficacy comparable to that of BK, effects of the former but not the latter were, however, reduced by NAC. An involvement of extracellular $H_2O_2$ is equally unlikely. In theory, $H_2O_2$ can be generated by dismutation of $O_2^-$ produced by NOS(Morikawa et al., 2003) and relaxes $K^+$-induced contraction with low potency. However, BK-induced relaxation of depolarized arteries was only partly reduced by DETCA and not modified by even a high concentration of exogenous catalase. Rather, a NOS-derived dilator compound that stimulates sGC, is distinct from NO and HNO, and resists inactivation by $O_2^-$ seems to be involved. This proposal for agonist-stimulated endothelium-dependent relaxation of depolarized patient pericardial arteries is comparable to the one described above for basal conditions. Observations with indomethacin suggest that such a mechanism is responsible for three quarters of the relaxation and a dilator prostaglandin for the remaining quarter.

Relaxing responses to BK in arteries contracted with ET-1 were on average and in contrast to depolarized

segments, not modified by L-NAME or ODQ in the absence and presence of indomethacin. They were shifted to endothelium-dependent hyperpolarizing responses that could not be attenuated by scavengers of NO or HNO. This shift is selectively induced by ET-1 because in our previous study of CVD patient pericardial resistance arteries, BK-induced relaxation of contractile responses to the $TXA_2$-analogue U46618, but not ET-1, were significantly attenuated by L-NAME and indomethacin(Leurgans et al., 2016). $H_2O_2$ did not play a major role because inhibition of SOD, which is an important albeit not exclusive source of the peroxide, had only a small effect and especially because exogenous catalase did not reduce the potency or efficacy of BK in the presence of ET-1. This seemingly contrasts with previous reports from several laboratories including our own(Beyer et al., 2017; Ellinsworth, Sandow, Shukla, Liu, Jeremy & Gutterman, 2016; Freed, Beyer, LoGiudice, Hockenberry & Gutterman, 2014; Leurgans et al., 2016; Matoba et al., 2000; Munoz et al., 2018; Schulz, Katunaric, Hockenberry, Gutterman & Freed, 2019; Shimokawa & Morikawa, 2005). We previously evaluated the effect of exogenous catalase during simultaneous inhibition of NOS, sGC, COX and endothelial $Ca^{2+}$-activated $K^+$-channels(Leurgans et al., 2016). Almost by definition, continuous presence of both L-NAME and indomethacin has been part of most studies of endothelium-dependent hyperpolarization and evaluations of involvement of endothelium-derived $H_2O_2$ using catalase. Indomethacin was absent in our present experiments. It may be of interest to analyze in future studies how cyclooxygenase products modulate the role of $H_2O_2$ as an endothelium-derived hyperpolarizing factor in the microvascular wall. Earlier conclusions were strengthened by us and others with imaging studies using dyes that were designed to demonstrate reactive oxygen species within cells and not in the extracellular space. In line with this, PEGylated-catalase is used in recent investigations of $H_2O_2$ in flow-induced endothelium-dependent dilatation of coronary and subcutaneous arterioles from patients with and without coronary artery disease(Beyer et al., 2017; Ellinsworth, Sandow, Shukla, Liu, Jeremy & Gutterman, 2016; Schulz, Katunaric, Hockenberry, Gutterman & Freed, 2019). Several other mechanisms have been proposed to mediate endothelium-dependent vasodilatation in addition to endothelium-derived prostaglandins, NO, HNO and $H_2O_2$(Ellinsworth, Sandow, Shukla, Liu, Jeremy & Gutterman, 2016; Shimokawa & Godo, 2020; Vanhoutte, Shimokawa, Feletou & Tang, 2017). These include alternative chemical mediators and electrical communication via heterocellular gap junctions. Although we did not investigate the latter, it can not explain that endothelium-dependent relaxation of depolarization-induced contraction could be inhibited by L-NAME and ODQ but not by a scavenger of NO or by an inhibitor of SOD.

To better define the molecular mechanisms underlying the observed profiles of endothelium-dependent vasodilatation, indirect pharmacological analyses of small number of vessels from the type of patients that we investigated will not suffice. The patients and their vessels varied considerably in several aspects. For instance, not only the amplitudes of the contractions and of the endothelium-dependent relaxations (Figure 1) but also the extent to which BK-induced relaxation could be inhibited by L-NAME (Figure 10), varied considerably between individual patients. As a result, a large number of experiments was needed to detect for instance i) a significant difference of the maximal relaxation between patients with and without coronary artery disease and ii) that the relaxing response in the presence of ET-1 was not significantly modified by L-NAME. Experimental animal research is here not an immediate or straightforward solution. To the best of our knowledge, no type of vessel from an animal model has been described with comparable vascular pharmacological properties. Future studies can focus on human resistance arteries that are more widely available and that can be harvested from groups of individuals with or without cardiovascular and non-cardiovascular disease. To this end, human subcutaneous and omental resistance arteries can be considered(Beyer et al., 2017; Munoz et al., 2018; Shimokawa & Morikawa, 2005; Zinkevich, Fancher, Gutterman & Phillips, 2017). Pharmacological experiments using a standardized protocol in vessels from many diverse individuals, can be of value provided that these individuals are characterized in detail in terms of not only their demographics and standard risk factors. Large numbers can form the basis of association studies. Selection of novel circulating factors can be hypothesis-driven and lead to confirmatory studies in dedicated experimental animals. These approaches were recently applied with respect to the role of a subtype of NADPH oxidases (NOX5) in the development of regional endothelial dysfunction and systolic hypertension with ageing(Elbatreek et al., 2020).

In resistance arteries from patients with CVD requiring surgery, we confirmed that endothelium-dependent relaxation can be inhibited by L-NAME and ODQ during depolarization-induced contraction but not in the presence of ET-1. A role for NO, HNO or $H_2O_2$ in these endothelium-dependent relaxations could not be confirmed. Alternative mechanisms seem to be involved. They do not depend on reactive oxygen species and are resistant to elevated levels of superoxide anions.

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Table 1. Patients' clinical information and medication.

| N | 53 |
|---|---|
| Age (years) | 67 (61-75) |
| Male (%) | 62 |
| Surgery: CABG/VRS/both (%) | 47/47/6 |
| Smoking (yes/former/no/unknown; %) | 19/48/15/17 |
| Body mass index ($kg \cdot m^{-2}$) | 27.0 ± 0.5 |
| Known hypertension (%) | 62 |
| Systolic blood pressure (mmHg) | 142 (127-150) |
| Diastolic blood pressure (mmHg) | 79 ± 1 |
| Type 2 diabetes mellitus (%) | 15 |
| Hyperlipidaemia (%) | 89 |
| Ejection fraction (%) | 60 (50-60) |
| HbA1c ($mmol \cdot mol^{-1}$) | 38 (36-41) |
| Plasma creatinine ($\mu mol \cdot L^{-1}$) | 88 (75-96) |
| Statins (%) | 71 |
| Low-dose aspirin (%) | 57 |
| Warfarin/P2Y antagonist (%) | 2/17 |
| ACEI/ARB (%) | 19/21?¿? |
| 2 anti-hypertensive drugs (%) | 58 |

Normally distributed data are shown as means ± SEM and non-normally distributed data are shown as median (IQR) or % of the study group. CAGB (coronary artery bypass grafting), VRS (valve replacement surgery), ACEI (angiotensin-converting enzyme inhibitor), ARB (angiotensin $AT_1$ receptor antagonist).

Table 2. Pharmacological tools.

| Chemical | Action | Concentration | References |
|---|---|---|---|
| 7-NI | Inhibitor of nNOS | 100 µM | (Moore, Wallace, Gaffen, Hart & Babbedge, 19 |
| Amitrole | Inhibitor of catalase | 50 mM | (Mian & Martin, 1997) |
| Catalase | Scavenger of extracellular $H_2O_2$ | 2000 U/mL | (Matoba et al., 2000) |
| c-PTIO | Scavenger of NO | 300 µM | (Akaike et al., 1993; Joseph, Kalyanaraman & |
| CXL-1020 | HNO-donor compound | 0.001-100 µM | (Sabbah et al., 2013; Zhu et al., 2015) |
| DETCA | Inhibitor of Cu/Zn superoxide dismutase | 3 mM | (Omar, Cherry, Mortelliti, Burke-Wolin & Wol |
| Indomethacin | Inhibitor of cyclooxygenases | 10 µM | (Vane, Bakhle & Botting, 1998) |
| L-NAME | Inhibitor of NO-synthases (NOS) | 100 µM | (Rees, Palmer, Schulz, Hodson & Moncada, 19 |
| NAC | Scavenger of HNO | 3 mM | (Ellis, Li & Rand, 2000; Pino & Feelisch, 1994; |

| Chemical | Action | Concentration | References |
|---|---|---|---|
| NPLA | Inhibitor of nNOS | 3 μM | (Cooper, Mialkowski & Wolff, 2000) |
| ODQ | Inhibitor of soluble guanylyl cyclase | 10 μM | (Garthwaite, Southam, Boulton, Nielsen, Schm |
| SNP | NO-donor compound | 0.001-1 μM | (Ignarro et al., 1980) |

**Figure legends**

Figure 1. Mean lumen diameter (A), contractile response to 32 mM $K^+$ (B), relaxing response to 1 μM bradykinin (C) and the relation of the $K^+$-induced contraction to the lumen diameter (D) in the pericardial resistance arteries investigated. Data are shown as mean of the observations in 3 – 6 arterial segments from the same patient (dots) and as median and interquartile range of the entire study group.

Figure 2. Relaxing responses to bradykinin (BK) during contraction stimulated by $K^+$ or endothelin-1 (ET-1) in preparations without (+Endo) and with (-Endo) prior gentle mechanical damage of their luminal surface. Mean observations (A) along with typical tracings of tension as a function of time illustrating effects of BK during contraction stimulated with $K^+$ (B) or ET-1 (C) in arterial segments from the same patient.

Figure 3. Immunohistochemical (IHC) staining for detection of NO-synthases and antioxidant enzymes in patient pericardial resistance arteries illustrated by typical examples and results of semi-quantitative analyses summarized by violin plots (N = 28). A and B, endothelial NO-synthase (eNOS) in a pericardial resistance artery and an atrial appendage, respectively. C, inducible NO-synthase (iNOS), D, neuronal NO-synthase (nNOS), E, catalase (CAT) and F, superoxide dismutase 1 (SOD1) in pericardial resistance arteries. EC, endothelium; SMC, medial smooth muscle; ADV, tunica adventitia. Scale bar 50 μm.

Figure 4. Effects of inhibition of prostanoid, oxidative and nitrosative processes on endothelium-independent SNP-induced relaxations in $K^+$-precontracted pericardial arteries. A and B, relaxing responses to 1 μM SNP in the absence and presence of 100 μM L-NAME, 300 μM c-PTIO and 10 μM ODQ in the absence (A) and presence of 10 μM indomethacin (INDO, B). C and D, cumulative-concentration response curves to SNP (C) and CXL-1020 (D) in the absence and presence of 3 mM NAC, 3 mM DETCA and 50 mM Amitrole. Data are shown as means ± SEM; n = 6-12. *, significantly different from control after comparison by one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test.

Figure 5. Effects of 2000 U/ml catalase (CAT) and 100 μM L-NAME on relaxing responses to $H_2O_2$ in pericardial arteries made to contract with 32 mM $K^+$(A) or an equieffective concentration of ET-1 (B). Data are shown as means ± SEM; n = 5-9. *, significantly different from control after comparison by one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test.

Figure 6. Effects of inhibition and scavenging of cyclooxygenase, nitrosative and oxidative pathways on contractile responses to 32 mM $K^+$ in pericardial resistance arteries. A, effect of 300 μM c-PTIO, 100 μM L-NAME and 10 μM ODQ in the absence (circles) and presence (rhombuses) of 10 μM indomethacin (INDO). B, effect of 3 mM NAC, 3 mM DETCA and 50 mM amitrole; C, 2000 U/ml CAT, 2000 U/ml CAT + 100 μM L-NAME and 100 μM L-NAME; and D, 100 μM 7-NI, 3 μM NPLA and 100 μM L-NAME on the contractile response in segments from the same arteries, respectively. Data are shown as mean ± SEM; N = 6-15. * , $P < 0.05$ significantly different from control; #, $P < 0.05$ compared to Control + INDO. Statistical significance of differences was assessed by one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test.

Figure 7. Effects of inhibition of cyclooxygenase, nitrosative and oxidative processes on BK-induced relaxations in pericardial resistance arteries precontracted with 32 mM $K^+$. A and B, relaxing responses to 1 μM BK in the absence and presence of 100 μM L-NAME, 300 μM c-PTIO and 10 μM ODQ in the absence (A, circles) and presence of 10 μM indomethacin (INDO) (B, rhombuses). C and D, cumulative-concentration response curves to BK in the absence and presence of 3 mM NAC, 3 mM DETCA and 50 mM Amitrole (C) or 2000 U/ml CAT, 2000 U/ml CAT + 100 μM L-NAME and L-NAME alone. Data are shown as means ± SEM; N

= 6-14. * , $P < 0.05$ versus control (one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test).

Figure 8. Effects of non-selective NOS inhibition and nNOS-selective inhibition on BK-induced endothelium-dependent relaxation in pericardial resistance arteries. Cumulative concentration response curves summarizing relaxing effects of BK in $K^+$- (A) and ET-1- (B) pre-contracted arteries in the absence and presence of 100 μM 7-NI, 3 μM NPLA or 100 μM L-NAME. Data are shown as means ± SEM; n = 5-11. *, P < 0.05 versus control (one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test).

Figure 9. Effects of inhibition of cyclooxygenase, nitrosative and oxidative processes on BK-induced relaxations in pericardial resistance arteries precontracted with ET-1. A and B, relaxing responses to 1 μM BK in the absence and presence of 100 μM L-NAME, 300 μM c-PTIO and 10 μM ODQ in the absence (A, circles) and presence of 10 μM indomethacin (INDO) (B, rhombuses). C and D, cumulative-concentration response curves to BK in the absence and presence of 3 mM NAC, 3 mM DETCA and 50 mM Amitrole (C) or 2000 U/ml CAT, 2000 U/ml CAT + 100 μM L-NAME and L-NAME alone. Data are shown as means ± SEM; N = 6-12. * , $P < 0.05$ versus control (one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test). These results were obtained after the same arterial segments were investigated for BK-induced relaxation during $K^+$-induced contraction (Figure 7).

Figure 10. Comparison between arteries from patients that required coronary bypass grafting (CABG, left) or valve replacement surgery (VRS, right). The individual data illustrate the effect of 100 μM L-NAME on the relaxing response to 1 μM BK in the same arterial segments made to contract with 32 mM $K^+$ or ET-1.

Figure 1

Figure 2

Figure 3

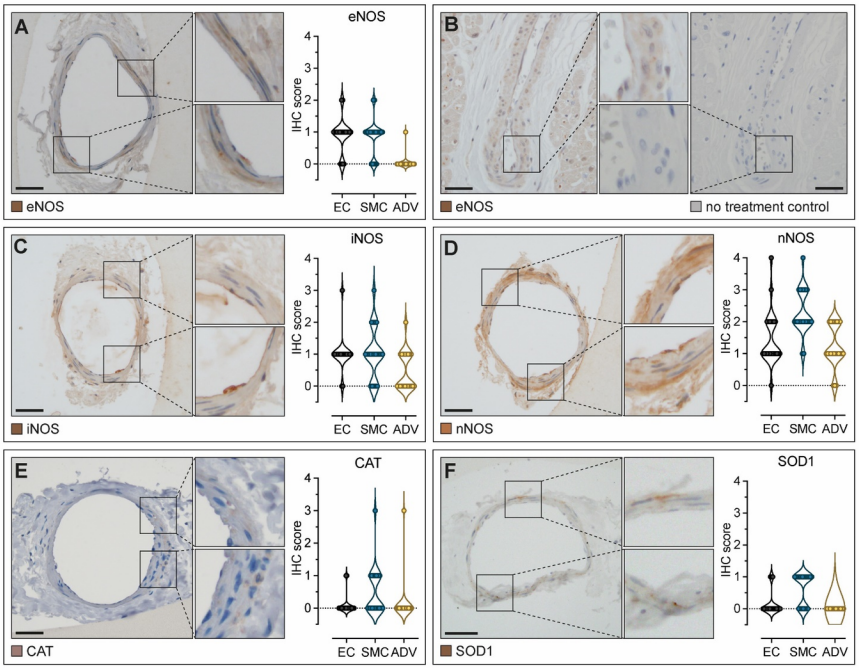



Figure 4

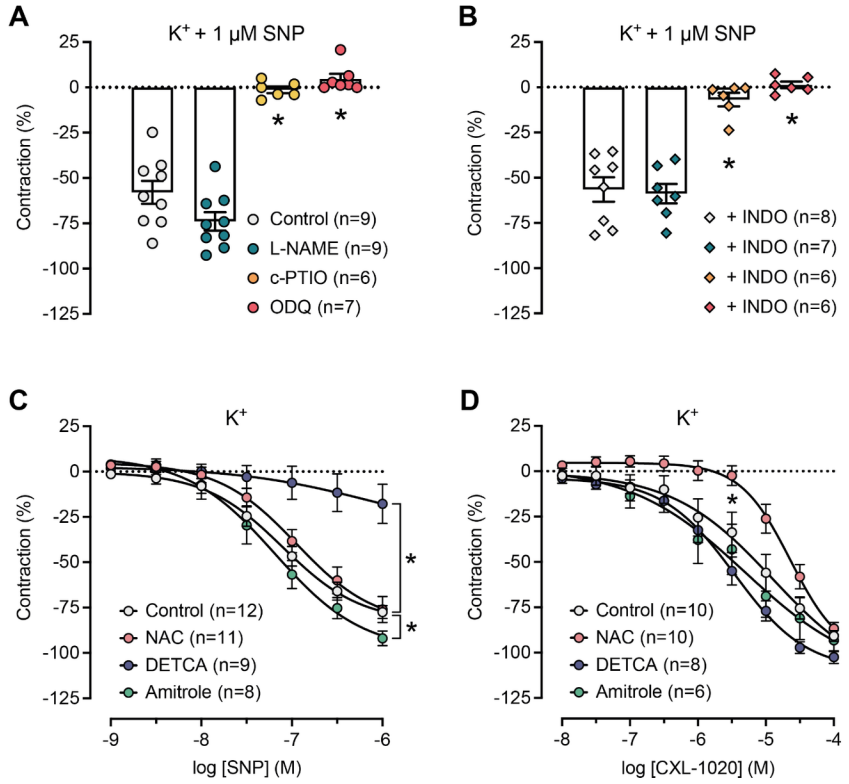



Figure 5

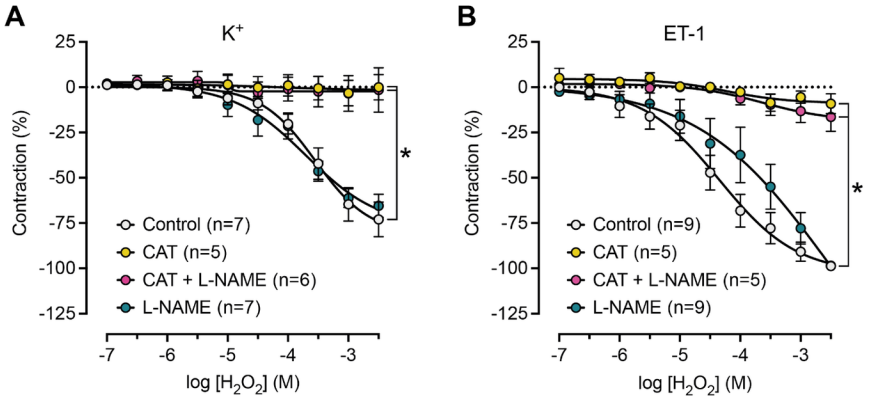



Figure 6.

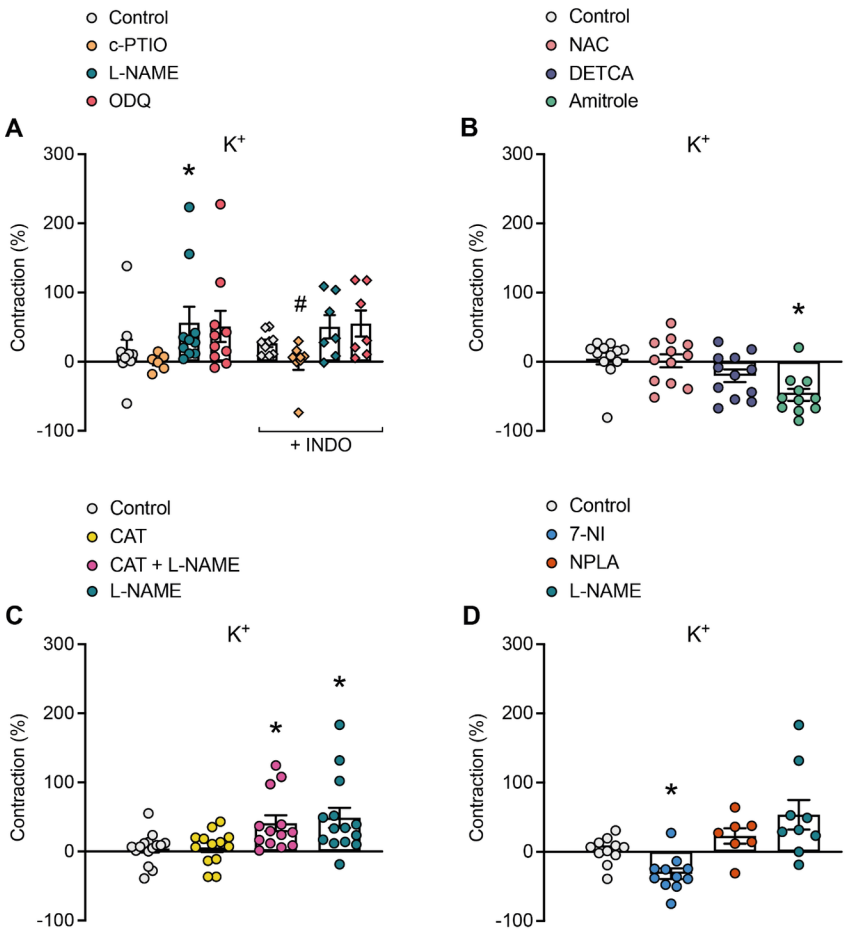



Figure 7

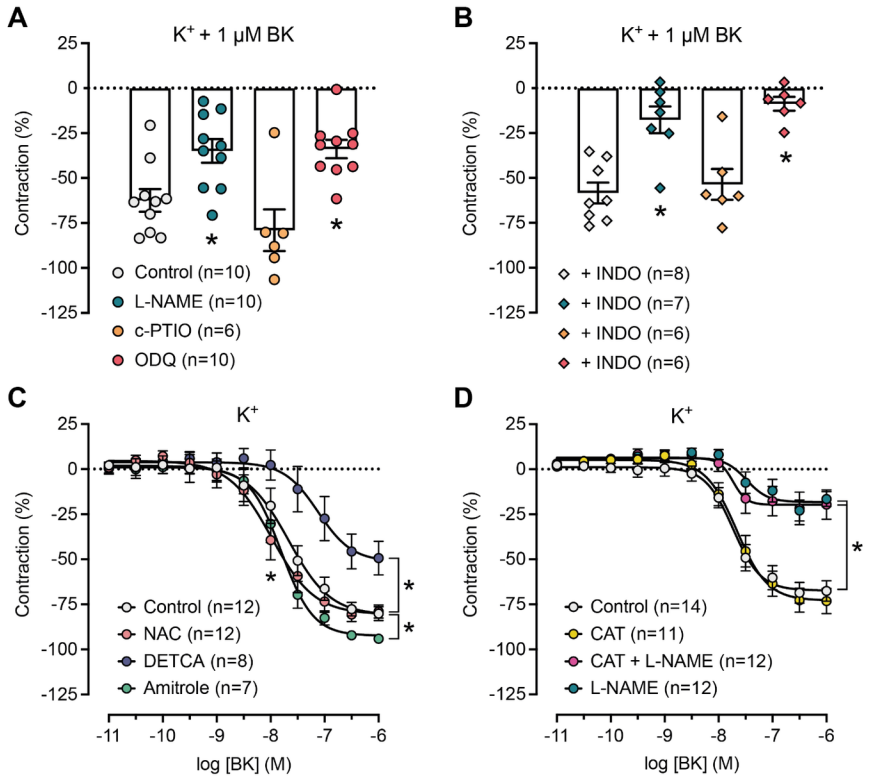



Figure 8

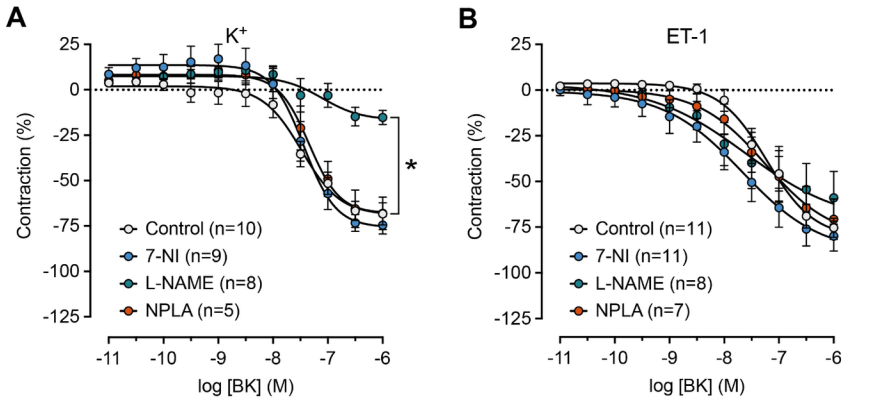



Figure 9

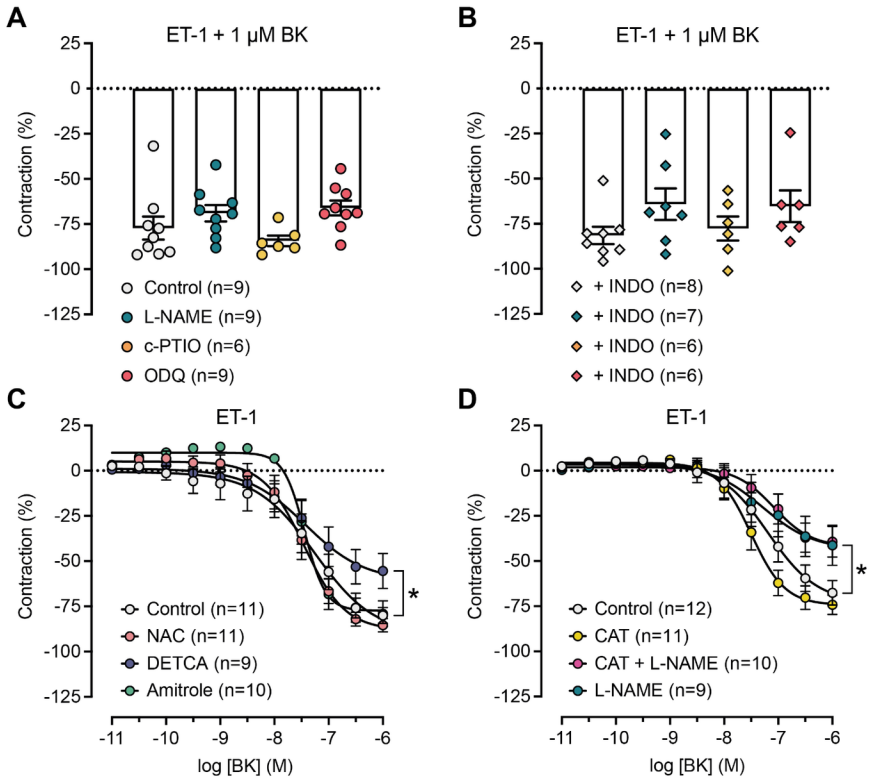
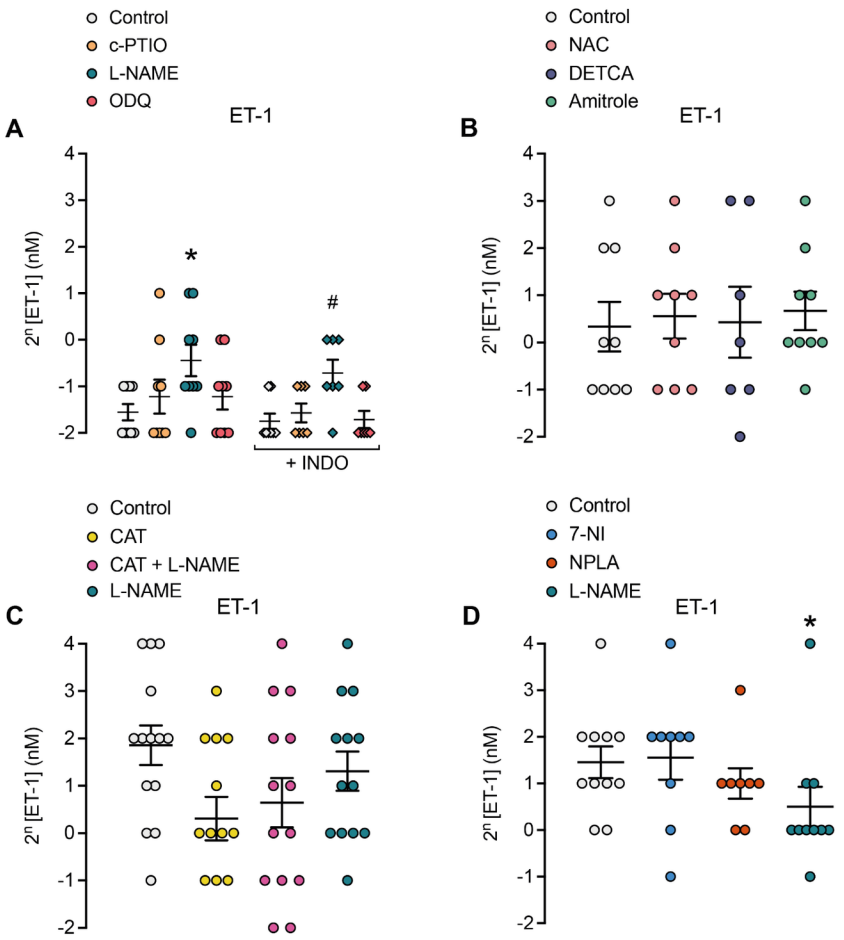



Figure 10

Supplemental Figure 1




Effects of inhibition of prostanoid, oxidative and nitrosative processes on the sensitivity to the contractile effect of ET-1 in pericardial resistance arteries. Individual data points show the concentration of ET-1 that was needed to cause a contraction of similar amplitude than that induced by 32 mM $K^+$. **A** , in the absence and presence of 100 µM L-NAME, 300 µM c-PTIO and 10 µM ODQ during incubation without or with 10 µM indomethacin (INDO). **B** , effects of 3 mM NAC, 3 mM DETCA and 50 mM amitrole; **C** , 2000 U/ml CAT, 2000 U/ml CAT + 100 µM L-NAME and 100 µM L-NAME; and**D** , 100 µM 7-NI, 3 µM NPLA and 100 µM L-NAME. Data are also shown as median and IQR; N = 6-15. * , $P < 0.05$ significantly different from control; #, $P < 0.05$ compared to Control + INDO. Statistical significance of differences was assessed by one-way mixed-effects ANOVA corrected for multiple comparisons by Dunett's post-hoc test.