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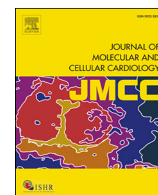

# Endothelin-1 impairs coronary arteriolar dilation: Role of p38 kinase-mediated superoxide production from NADPH oxidase

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ABSTRACT

Elevated levels of endothelin-1 (ET-1), a potent vasoactive peptide, are implicated as a risk factor for cardiovascular diseases by exerting vasoconstriction. The aim of this study was to address whether ET-1, at sub-vasomotor concentrations, elicits adverse effects on coronary microvascular function. Porcine coronary arterioles (50–100 μm) were isolated, cannulated and pressurized without flow for in vitro study. Diameter changes were recorded using a videomicrometer. Arterioles developed basal tone (60 ± 3 μm) and dilated to the endothelium-dependent nitric oxide (NO)-mediated vasodilators serotonin (1 nmol/L to 0.1 μmol/L) and adenosine (1 nmol/L to 10 μmol/L). Treating the vessels with a clinically relevant sub-vasomotor concentration of ET-1 (10 pmol/L, 60 min) significantly attenuated arteriolar dilations to adenosine and serotonin but not to endothelium-independent vasodilator sodium nitroprusside. The arteriolar wall contains $ET_A$ receptors and the adverse effect of ET-1 was prevented by $ET_A$ receptor antagonist BQ123, the superoxide scavenger Tempol, the NADPH oxidase inhibitors apocynin and VAS2870, the NOX2-based NADPH oxidase inhibitor gp91 ds-tat, or the p38 kinase inhibitor SB203580. However, $ET_B$ receptor antagonist BQ788, $H_2O_2$ scavenger catalase, scrambled gp91 ds-tat, or inhibitors of xanthine oxidase (allopurinol), PKC (Gö 6983), Rho kinase (Y27632), and c-Jun N-terminal kinase (SP600125) did not protect the vessel. Immunohistochemical staining showed that ET-1 elicited Tempol-, apocynin- and SB203580-sensitive superoxide productions in the arteriolar wall. Our results indicate that exposure of coronary arterioles to a pathophysiological, sub-vasomotor concentration of ET-1 leads to vascular dysfunction by impairing endothelium-dependent NO-mediated dilation via p38 kinase-mediated production of superoxide from NADPH oxidase following $ET_A$ receptor activation.

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## 1. Introduction

Endothelin-1 (ET-1) is an endogenous vasoactive peptide that exerts robust and prolonged vasoconstriction of coronary arteries, with reported half maximal effective concentration ($EC_{50}$) in the nanomolar range from various mammalian species, including mice (~1.0 nmol/L) [1], rats (~12.5 nmol/L) [2,3], dogs (~12.5 nmol/L) [3], pigs (~6.7 nmol/L) [4,5], monkeys (~181 nmol/L) [3], and humans (~3.5 nmol/L) [6,7]. The extent of coronary vasoconstriction to ET-1 appears to be inversely related to vessel size [8,9]. The normal circulatory level of ET-1 in the peripheral blood is about 1–3 pmol/L (2–8 pg/mL) [10–15]. In patients with hypertension or ischemic heart disease, the plasma level of ET-1 rises 2- to 4-fold [10–15] and the expression of endothelin type A ($ET_A$) and type B ($ET_B$) receptors mRNA in coronary arteries was significantly higher [16]. An increased circulating level of ET-1 is generally associated with poor clinical outcome and survival rate in patients with myocardial infarction [17–20] and is regarded as an independent predictor of myocardial no-flow, reduced left ventricular function, and long-term mortality [17].

The mechanism by which ET-1 exerts its adverse effect on the cardiovascular system has been suggested to involve severe vasoconstriction and/or the disturbance of vascular redox balance. Accordingly, infusion of ET-1 (1.5 nmol/L) potentiates the increase in coronary resistance in the hearts isolated from spontaneous hypertensive rats in a manner sensitive to the administration of nitric oxide (NO) precursor L-arginine. It is likely that the vasoconstriction elicited by ET-1 may compromise the coronary NO-mediated vasodilator function in this disease model [2]. In addition, exposure of the conduit vasculature to high levels of ET-1 (0.1 and 1 nmol/L) leads to increased superoxide anion production in the vessel wall [21] and may consequently contribute to ET-1-mediated vasoconstriction. However, it is unclear whether ET-1 can influence vasodilation elicited by the endothelial NO in the intact coronary microvasculature without the participation of its vasoconstrictor action. In the present study, we addressed whether a pathophysiological, yet sub-vasomotor concentration of ET-1 is capable of exerting oxidative stress and an

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adverse effect on endothelium-mediated NO function in small coronary arterioles, which control and regulate resistance and flow in the heart [22]. More specifically, we examined the endothelium-dependent NO-mediated dilation of porcine coronary arterioles in the absence and presence of a sub-vasomotor concentration of ET-1 and investigated the relative contribution of superoxide generating enzymes and stress-activated protein kinases to the pathophysiological effect of ET-1 on coronary vasomotor function.

## 2. Materials and methods

### 2.1. Materials

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). All animal procedures were approved by the Scott & White Institutional Animal Care and Use Committee, and have been described in detail previously [23]. In brief, pigs (8 to 12 weeks old of either sex; 8–14 kg) were anesthetized with Telazol® (4 mg/kg, intramuscular injection) and maintained at a surgical plane with 2–3% isoflurane inhalation. The heart was excised and placed in ice-cold saline. Subepicardial arterioles (1 mm in length; 40–80 μm in internal diameter in situ) were dissected out for in vitro study [23]. Coronary arterioles were cannulated with glass micropipettes and pressurized to 60 $cmH_2O$ intraluminal pressure and bathed in physiological salt solution (PSS) at 37 °C. The internal diameter of the coronary arteriole was measured using videomicroscopic techniques [23].

### 2.2. Functional assessment of isolated coronary arterioles

To characterize the vasomotor response to ET-1, concentration-dependent constriction of isolated coronary arterioles to ET-1 (1 pmol/L to 10 nmol/L) was assessed at 5 min after ET-1 administration. To examine the impact of a sub-vasomotor concentration of ET-1 on NO-mediated vasodilation, concentration-dependent responses of coronary arterioles to endothelium-dependent NO-mediated vasodilators serotonin (0.1 nmol/L to 0.1 μmol/L) [24] and adenosine (0.1 nmol/L to 10 μmol/L) [24] were examined in the absence (vehicle treatment) and presence of a normal (1 pmol/L) or clinical relevant (10 pmol/L) concentration of ET-1 for 60 min. Our preliminary data indicated that treatment of ET-1 for 30 min did not consistently inhibit coronary arteriolar dilations to serotonin and adenosine. In contrast, ET-1 treatment for 60 min consistently reduced coronary arteriolar dilations to serotonin and adenosine and this inhibitory effect was not further enhanced at 120 min. Therefore, a 60-minute treatment was chosen for further study. To test whether ET-1 has a direct effect on the vasodilator machinery of smooth muscle, the ET-1-treated vessels were challenged with the endothelium-independent vasodilator sodium nitroprusside (SNP), which has been shown to lack reactivity with superoxide [25] and to elicit vasodilation via activation of guanylyl cyclase through formation of nitrosonium cation [25–27].

The roles of ET receptor subtypes, $ET_A$ and $ET_B$, in mediating the ET-1 effect were assessed by co-incubating the vessels with ET-1 (10 pmol/L) and $ET_A$ receptor blocker BQ-123 and $ET_B$ receptor blocker BQ-788, respectively, for 60 min. The vasodilations to serotonin and adenosine were subsequently assessed. To examine whether the effect of ET-1 is affected by the inhibition of NO synthase (NOS), the vasodilations to serotonin and adenosine were examined in the presence of ET-1 and NOS inhibitor L-NAME (10 μmol/L) [24]. The involvements of superoxide, hydrogen peroxide ($H_2O_2$) and peroxynitrite in the adverse effect of ET-1 were also examined by co-incubating the vessels with ET-1 plus superoxide scavengers Tempol (1 mmol/L) [28,29] or MnTBAP (10 μmol/L) [30], $H_2O_2$ scavenger catalase (1000 units/mL) [31], and peroxynitrite scavenger urate (0.1 mmol/L) [29] for 60 min, respectively. To determine the contribution of superoxide generation enzyme NADPH oxidase (NOX) in mediating the ET-1 effect, vasodilations to serotonin and adenosine were studied in separate groups of vessels treated with ET-1 (0.1 nmol/L) in combination with NOX inhibitor apocynin (100 μmol/L) [28], VAS2870 (10 μmol/L) [32], NOX2-based assembly inhibitor gp91 ds-tat (10 μmol/L) [33] or Rac1/NOX2 inhibitor mycophenolic acid (10 μmol/L, Santa Cruz Biotechnology) [34]. The role of xanthine oxidase was investigated using its inhibitor allopurinol (10 μmol/L) [35]. The involvements of protein kinase C (PKC) and c-Jun N-terminal kinase (JNK) were examined by treating another groups of vessels with ET-1 combined with inhibitors for PKC (Gö 6983, 1 μmol/L) [36] and JNK (SP600125, 5 μmol/L; Calbiochem) [37], respectively. The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (0.1 μmol/L; Calbiochem) [38] and Rho kinase (ROCK) inhibitors Y27632 (0.1 μmol/L; Calbiochem) [39] or H1152 (0.1 μmol/L; Tocris Bioscience) [40] were administered to examine the roles of these kinases in mediating the effects of ET-1. The SP600125, VAS2870, SB203580, H1152, and Gö 6983 were dissolved as stock solutions in dimethyl sulfoxide, which had final concentrations of 0.01%, 0.03%, 0.001%, 0.01%, and 0.01% by volume in the vessel bath, respectively. The mycophenolic acid was dissolved in ethanol (0.05% final bath concentration). The final concentrations of these solvents had no effect on vasomotor function. All drugs, unless otherwise stated, were obtained from Sigma and were dissolved in PSS.

### 2.3. Immunohistochemical analysis of isolated coronary arterioles

To identify and localize vascular $ET_A$ receptors, coronary arterioles were embedded and frozen in OCT compound (Tissue-Tek) [41]. Frozen sections (10-μm thick) were fixed in 4% paraformaldehyde, and then immunolabeled with specific primary antibodies for $ET_A$ receptors (Sigma) and endothelial NOS (eNOS; Santa Cruz Biotechnology). Afterwards, the slides were incubated with rhodamine red-labeled (Jackson Laboratories) and FITC-labeled (Jackson Laboratories) secondary antibodies. Staining control tissues were exposed for the same duration to non-immune serum (Jackson Laboratories) in place of primary antibody. Slides were observed for red (rhodamine red for eNOS) and green (FITC for $ET_A$) images, and analyzed using a fluorescence microscope (Axiovert 200, Zeiss). Merged images were created with ImageJ software.

### 2.4. Detection of vascular superoxide

Superoxide production in isolated coronary arterioles was evaluated with the fluorescent dye dihydroethidium (DHE) as described previously [28,38]. In this series of studies, coronary arterioles (40 to 100 μm in diameter and 1.5 mm in length) were incubated with PSS vehicle or ET-1 (10 pmol/L) in combination with Tempol (1 mmol/L), apocynin (100 μmol/L), VAS2870 (10 μmol/L), SB203580 (0.1 μmol/L) or Y27632 (0.1 μmol/L), and then stained with DHE (4 μmol/L) for 30 min. After being washed, arterioles were embedded in OCT compound (Tissue-Tek) for cryostat sections. The embedded arterioles were cut into 10-μm-thick sections and placed on glass slides. The DHE fluorescence image was taken at excitation/emission wavelength of 360/460 nm with a fluorescence microscope (Axiovert 200, Zeiss). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental samples.

### 2.5. Data analysis

As described previously, coronary vasomotor response to the ET-1 was normalized to resting vessel diameter following development of basal tone [42]. Vascular response to vasodilators was normalized to the maximal diameter changes in response to 100 μmol/L SNP and expressed as a percentage of maximal dilation [43]. The vascular DHE fluorescence images were quantitatively analyzed after subtracting the background fluorescence using ImageJ software (National Institutes of

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