Life Sciences 91 (2012) 723–728

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Endothelin-1 increases superoxide production in human coronary artery bypass grafts

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ARTICLE INFO

Article history:
Received 31 October 2011
Accepted 28 February 2012

Keywords:
Endothelin-1
Superoxide
Coronary bypass grafts
Endothelial dysfunction

ABSTRACT

Aims: Endothelin-1 (ET-1) has been shown to increase endothelial superoxide (O_2^-) production in experimental animal models. It is unclear whether ET-1 increases O_2^- production in humans. We sought to elucidate whether ET-1 increases O_2^- production in human vessels and to identify the mechanism behind this effect.
Main methods: Segments of internal mammary artery (IMA) and human saphenous vein (HSV) were harvested from 90 patients undergoing elective coronary artery bypass graft surgery. Paired vessel rings were incubated in the presence and absence of ET-1 (10^{-10} M), the ET_A receptor antagonist BQ123 alone, or in combination with the ET_B receptor antagonist BQ788 (dual BQ) and known inhibitors of sources of O_2^- and further analysed for O_2^- production using lucigenin-enhanced chemiluminescence and DHE fluorescence.
Key findings: ET-1 increased O_2^- production in both IMA (2.6 ± 1.5 vs. 1.4 ± 0.8 relative light units/s/mg tissue (RLU); $n = 33$; $p < 0.0001$) and HSV (1.4 ± 0.8 vs. 1.1 ± 0.6 RLU; $n = 24$; $p < 0.05$). The increase in O_2^- production induced by ET-1 in IMA was inhibited by co-incubation with dual BQ ($p < 0.05$; $n = 15$) and BQ123 ($p < 0.05$; $n = 17$). Of known O_2^- inhibitors, only incubation with Tiron and diphenyleneiodonium resulted in a significant reduction in ET-mediated O_2^- production.
Significance: ET-1 increases O_2^- production especially in human arteries and less so in veins from patients with coronary artery disease via a receptor-dependent pathway involving a flavin dependent enzyme which is likely to be NADPH oxidase. Production of O_2^- may be an important factor underlying the negative effects of ET-1 on vascular function such as impairment of endothelium-dependent vasodilatation and pro-inflammatory effects.

Introduction

The development of atherosclerosis occurs in the presence of risk factors such as diabetes, hypertension and hypercholesterolemia. These vascular disease states present a disturbed internal balance between vasodilator and vasoconstrictor activity and an increase in the production of reactive oxygen species (ROS) (Munzel et al., 2008). ROS naturally exist in the vessel wall and act as intracellular signalling molecules influencing cell growth (Buetler et al., 2004). When ROS production exceeds the antioxidant defences, pathological effects of ROS are seen, which may be the case following reduced bioavailability of nitric oxide (NO) due to rapid chemical inactivation (Ignarro et al., 1987). Reduced bioavailability of NO is associated with increased levels of the vasoconstrictive peptide endothelin-1 (ET-1) that contribute to endothelial dysfunction in atherosclerosis (Böhm et al., 2002b; Lerman et al., 1991, 1995). ET-1 is predominantly produced by vascular endothelial cells and exerts its effects through binding to two G-protein coupled membrane receptors: ET_A and ET_B. A possible link between ET-1 and ROS production has been suggested. ET-1 stimulates ROS production in human endothelial and vascular smooth muscle cell cultures (VSMC) (Dong et al., 2005; Duerrschmidt et al., 2000), as well as in different rat (Li et al., 2003b; Loomis et al., 2005; Lopez-Sepulveda et al., 2010) and mouse models (Li et al., 2003a). Both ET_A (Li et al., 2003b) and ET_B (Dong et al., 2005; Duerrschmidt et al., 2000) receptors have been suggested to contribute to superoxide (O_2^-) production from different sources. However, there is limited understanding regarding the effect of ET-1 on ROS production in human vessels. We have previously shown that ET-1 infused in the forearm of patients with coronary artery disease (Böhm et al., 2002a) and healthy individuals (Böhm et al., 2007) causes endothelial dysfunction. This impairment could in healthy individuals be successfully inhibited by the antioxidant vitamin C suggesting a possible involvement of ET-induced O_2^- production. However, no measurement of ROS production was performed. Thus, the effect of ET-1 on ROS production in intact human blood vessels remains to be investigated. Therefore, the aim of the present study was to investigate; (1) whether ET-1 increases O_2^- production in human vessels, (2) if there exist differences in ET-induced O_2^-

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0024-3205
doi:10.1016/j.lfs.2012.03.024

production between human arteries and veins, (3) which ET receptor that is involved in ET-induced O_2^- production, and (4) which source of O_2^- that is predominantly involved in ET-induced O_2^- production.

Material and methods

Study subjects

We recruited 90 patients with CAD undergoing coronary artery bypass grafting (CABG) at the John Radcliffe Hospital, Oxford, UK. Inclusion criteria were coronary artery disease in need of elective or subacute CABG and exclusion criteria were emergency CABG and unwillingness to participate. Each patient gave oral and written informed consent. The study was performed in accordance with the Declaration of Helsinki. The study was approved by the local Research Ethics Committee. Basal characteristics of the patients are presented in Table 1.

Tissue samples

Samples of internal mammary artery (IMA, n = 73) and saphenous vein (HSV, n = 24) were obtained at the time of CABG. The vessel segments were dissected free from surrounding tissue, rinsed from blood and then quickly transported from surgery in ice-cold oxygenated Krebs-Henseleit buffer to the laboratory.

Determination of vascular superoxide production

Vascular O_2^- production was measured in paired segments of IMA and HSV with the use of lucigenin-enhanced chemiluminescence (Guzik and Channon, 2005; Guzik et al., 2002). The protocol has been validated and at the low dose of 5 μM lucigenin no redox cycling has been detected (Dikalov et al., 2007; Guzik and Channon, 2005). The segments were divided into 4–6 rings, each approximately 3 mm thick, depending on size, weight ranging from 6 to 20 mg/ring. The vessels were opened longitudinally to expose the endothelial surface and equilibrated in oxygenated (95% O_2/5% CO_2) Krebs-HEPES buffer (pH 7.4) at 37 °C with and without ET-1 (10^{-10} M) for 45 min. The incubation time was chosen based on our previous studies showing marked reduction in endothelium-dependent vasodilatation in the human forearm following 30–60 min infusion of ET-1 (Böhm et al., 2002a, 2007; Lerman et al., 1995). The vessel segment was then quickly transferred to a luminometer containing low-concentration lucigenin (5 μmol/L) in order to measure O_2^- production. In subgroups, a dose-ranging study for ET-1 (10^{-11} to 10^{-8} M) was performed. Furthermore, additional rings were analysed after 20 min preincubation with either the ET_A receptor antagonist BQ123 (10^{-6} M) alone, or in combination with the ET_B receptor antagonist BQ788 (10^{-6} M; dual BQ), the inhibitor of flavin dependent enzymes diphenyleneiodonium (DPI, 5×10^{-5} M), the inhibitor of nitric oxide synthase L-nitro arginine methyl ester (L-NAME; 10^{-4} M), the inhibitor of xanthine oxidase oxypurinol (10^{-5} M), the inhibitor of mitochondrial ROS rotenone (10^{-4} M), the superoxide scavenger Tiron (10^{-6} M) and the inhibitor of the assembly of NADPH oxidase apocynin (5×10^{-4} M). Following this preincubation, ET-1 was added for 45 min in each subgroup.

Table 1
Individual characteristics of patients.

No of patients	90
Men/women	78/12
Age (y, mean ± SD)	68 ± 8
Risk factors, n (%)	
Hypertension	63 (70)
Hypercholesterolemia	66 (73)
Smokers/exsmokers	14/46
Diabetes mellitus	26 (28)
Type II	23 (89)
Family history	43 (48)
Body mass index, kg/m^2	28 ± 4
Elective/acute surgery	
Angiographic extent of CAD, n (%)	82/8
1-vessel disease	2 (2)
2-vessel disease	19 (21)
3-vessel disease	69 (77)
Medication, n (%)	
Statins	79 (88)
Angiotensin-converting enzyme inhibitors	37 (41)
Angiotensin receptor blockers	16 (18)
Calcium channel blockers	21 (23)
Betablockers	63 (70)
Nitrates	35 (39)
Aspirin	71 (79)
Clopidogrel	20 (22)
Diuretics	7 (8)

Values are expressed as mean ± SD.

Oxidative fluorescent microphotography

In situ O_2^- production was determined in vessel cryosections with the oxidative fluorescent dye dihydroethidium (DHE) as previously described (Bendall et al., 2007). Paired vessel segments were incubated in the presence or absence of ET-1 (10^{-10} M) in Krebs-HEPES buffer for 45 min at 37 °C and then snap frozen in Tissue Tek OCT. Cryosections (30 μm) were equilibrated in the presence or absence of ET-1 for 30 min at 37 °C and then exposed to DHE (2 μmol/L) for 5 min. Fluorescence images of the endothelium (40x, Zeiss LSM 510 META laser scanning confocal microscope) were obtained from each vessel quadrant. Segments of vessel rings (with and without ET-1, n = 3) were analysed in parallel with identical imaging parameters. DHE fluorescence was quantified by automated image analysis with Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA).

Substances

ET-1, BQ123 and BQ788 (NeoMPS, Strasbourg, France) were all dissolved in Millipore filtered water and stored frozen at −20 °C. Apocynin, oxypurinol, rotenone, Tiron, DPI and L-NAME (Sigma Aldrich, UK), were dissolved according to the manufacturer's instructions and stored frozen at −20 °C. DHE (Invitrogen, UK), was dissolved in DMSO according to manufacturer's instructions.

Statistical analysis

Data are expressed as means ± SD if not stated otherwise. All variables were tested for normal distribution with the use of D'Agostino–Pearson normality test. Statistical differences were calculated by using Students paired *t*-test for between group comparisons. All statistical analyses were performed using Graph Pad 4.0 Prism Plus software.

Results

ET-mediated O_2^- production

A dose-ranging study revealed a marked increase in O_2^- production by ET-1 in IMA already from 10^{-10} M (Fig. 1a). This dose was therefore used in the subsequent studies. ET-1 induced a significant increase in O_2^- production in both IMA (Fig. 1b) and HSV (Fig. 1c). ET-1 had a significantly more pronounced effect on O_2^- production in IMA than in HSV (Fig. 1d).

In addition to O_2^- production measured by chemiluminescence, DHE staining of cryosections of IMA was performed in order to histologically visualize the possible intracellular source of ET-mediated O_2^- production. Fig. 2 shows DHE-stained cryosections of control and ET-treated IMA. The integrity of the vessel was kept with intact basal

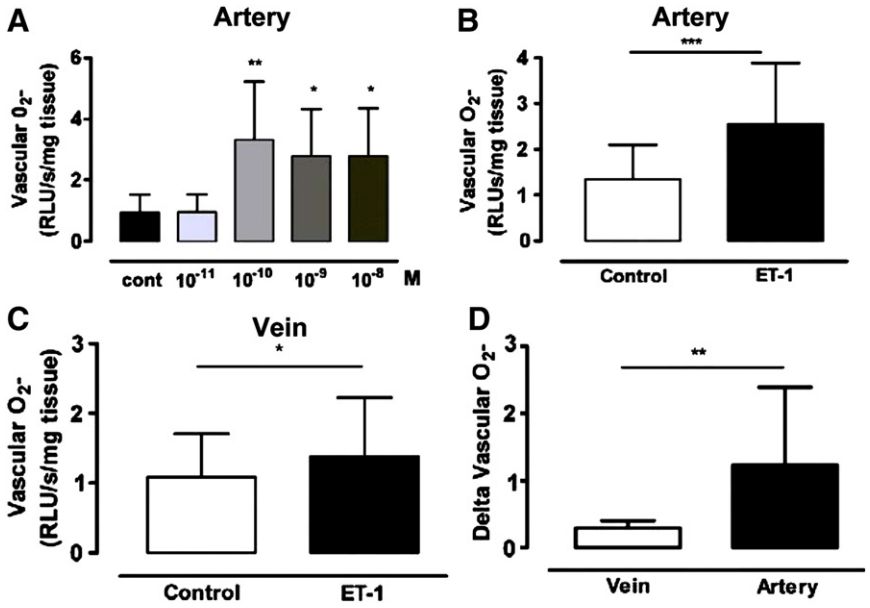

Fig. 1. (a): dose ranging study of ET-1 showing effect of 45 min ET-1 (0.01–10 nM) incubation on O_2^- production as determined by lucigenin-enhanced chemiluminescence in paired internal mammary arteries, n = 8. (b): effect of ET-1 (0.1 nM) on O_2^- production in paired internal mammary arteries (n = 33) and (c): in paired saphenous veins (n = 24). (d) Delta increase of vascular O_2^- production in internal mammary arteries and saphenous veins. Data are presented as means and SD; *p<0.05, **p<0.001, ***p<0.0001.

lamina. Sublaminally there was an increase of fluorescent signal coming from spindle shaped cells in ET-treated cryosections suggesting that a predominant fraction of ET-mediated O_2^- production originates from vascular smooth muscle cells.

ET-1 mediates O_2^- production via a receptor-dependent mechanism

Since the O_2^- production stimulated by ET-1 was significantly greater in IMA than in HSV, IMA was chosen for further mechanistic studies of ET-induced O_2^- production. To investigate whether ET-1 mediate O_2^- production via a receptor-dependent mechanism and to determine which receptor is most important for mediating O_2^- production, paired IMA rings were incubated in the presence of ET-1 and the selective ET_A receptor antagonist BQ123 or dual ET_A/ET_B blockade. The increase in O_2^- production induced by ET-1 in IMA was markedly inhibited by pre-incubation with BQ123 ($p<0.05$; Fig. 3) as well as by dual BQ ($p<0.05$; Fig. 3).

Sources of ET-mediated O_2^- in human vessels

In order to investigate through which enzymatic source ET-1 mediates its effects on O_2^- production in IMA, we incubated paired IMA segments with inhibitors of known O_2^- sources such as xanthine oxidases (oxypurinol), mitochondrial ROS (rotenone), eNOS uncoupling (L-NAME), and NADPH oxidases (DPI and apocynin). To confirm the results we also incubated with the superoxide scavenger Tiron. Incubation with Tiron and DPI (Fig. 4a) could significantly inhibit ET-induced O_2^- production. Incubation with apocynin, oxypurinol, rotenone and L-NAME had no effect on O_2^- levels stimulated by ET-1 (Fig. 4b).

Discussion

The main findings are that (1) ET-1 induces a significant increase in O_2^- in human arteries and veins used for CABG, (2) ET-induced O_2^- production is more pronounced in IMA than in HSV, (3) ET-mediated O_2^- production occurs via a receptor-dependent mechanism, predominantly via the ET_A receptor and (4) ET-induced O_2^- production is dependent on NADPH oxidase.

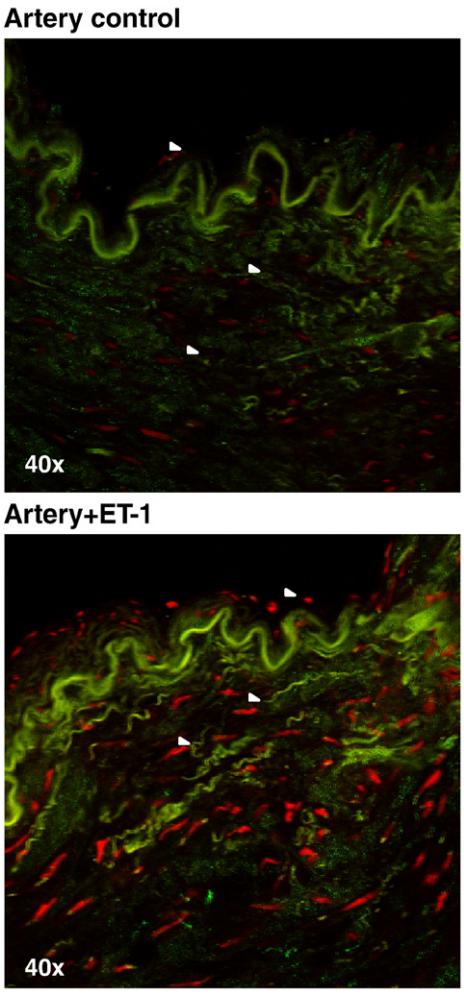

Fig. 2. Effect of 45 min ET-1 (0.1 nM) incubation on O_2^- production in a representative example of paired internal mammary arteries (n = 3) as determined by DHE fluorescence (red staining indicated with white arrows, green staining represents basal autofluorescence), 40× magnification.

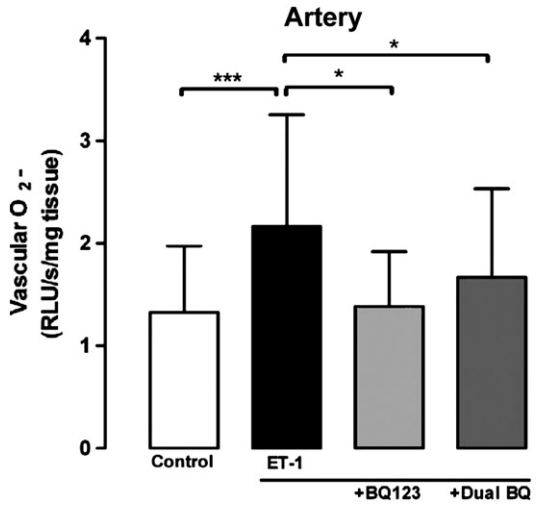

Fig. 3. Effect of 45 min ET-1 (0.1 nM) incubation on O_2^- production in paired internal mammary arteries with and without 20 min pre-incubation of BQ123 (10 μM) alone (n = 15) and in combination with BQ788 (10 μM; n = 17). Data are presented as means and SD; *p<0.05.

Previous studies have shown that ET-1 induces increased ROS production and is involved in initiating and maintaining endothelial dysfunction (Schiffrin, 2001). Furthermore, ET receptor blockade has been shown to improve endothelial function in human coronary arteries (Verma et al., 2001). However, there are no studies that have determined whether ET-1 stimulates O_2^- production in human arteries and the pathophysiological link to endothelial dysfunction remains unknown. In this study we therefore first sought to investigate whether exogenous ET-1 increases ROS production in human vessels from patients with CAD. ET-1 produced a significant increase in O_2^- production with a clear effect at 10^{-10} M. This concentration of ET-1 is considerably lower than those in previously presented studies on ET-induced O_2^- production (Duerrschmidt et al., 2000; Ergul et al., 2005; Loomis et al., 2005; Touyz et al., 2004) which have used concentrations ranging from 10^{-6} to 10^{-9} M. Several studies have shown that patients with CAD have increased levels of circulating ET-1 (Hedman et al., 2007; Sabatine et al., 2012; Yip et al., 2005). These levels are usually in picomolar range and its biological effect is unclear since secretion of ET-1 predominantly occurs abluminally. ET-1 immunoreactivity expressed in histological staining has been studied in vascular tissue from patients with CAD showing an increased percentage of positive staining particularly in mammary arteries (Sutherland et al., 2006) but to our knowledge no attempts have been made to measure the actual concentrations of intracellular ET-1 in these tissues and also study functional outcomes. We conclude that mammary arteries are not ET-1 naive and at low concentrations of exogenous ET-1 clear effects on ROS generation can be seen. Future studies would need to further investigate the correlation of intracellular levels of ET-1 and ROS generation. In this study we observed differences in ET-1 induced O_2^- production between IMA and HSV. This may be due to difference in structure, with HSV having more adventitia and less VSMC than IMA, but also differences in vascular contractility to ET-1, ET receptor expression and level of endothelin converting enzyme activity between the two vessel types (Heigl et al., 2002). In contrast we could not observe a difference in basal levels of O_2^- production between IMA and HSV i.e. prior to ET-1 stimulation suggesting that intrinsic differences became apparent on outcome only when ET-1 was added to the vessels.

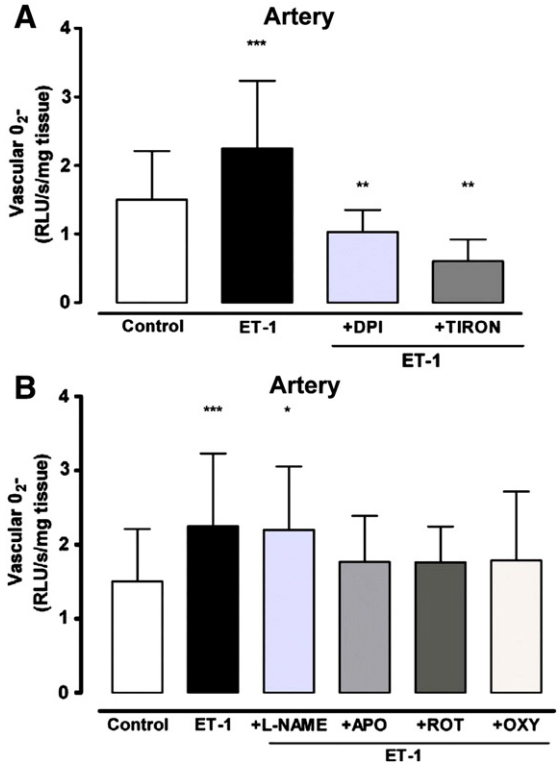

Fig. 4. Effect of 45 min ET-1 (0.1 nM) incubation on O_2^- production in paired internal mammary arteries with and without 20 min pre-incubation with (a) Tiron: n = 6 and DPI: n = 9, (b) rotenone: n = 7, L-NAME: n = 14, oxypurinol: n = 7, apocynin: n = 7. Data are presented as means and SD; **p<0.001.

In order to investigate whether ET-1 mediates O_2^- production via a receptor-dependent pathway we exposed paired segments of IMA to ET-1 in combination with its receptor antagonists BQ123 (ET_A) alone or in combination with BQ788 (ET_B). In vitro studies show that both receptors can contribute to O_2^- production (Dong et al., 2005; Li et al., 2003b). In our study we demonstrate a clear reduction of ET-induced O_2^- production by selective ET_A receptor blockade and no additional effect of dual ET_A/ET_B receptor blockade. These observations suggest that ET_A is the predominant receptor mediating arterial ROS production. Activation of ET_B receptors expressed on VSMC results in vasoconstriction, cell proliferation and superoxide production (Böhm and Pernow, 2007). In contrast, activation of ET_B receptors on endothelial cells leads to release of NO which may reduce O_2^- bioavailability (d'Uscio et al., 2000). According to our results, the net effect of blocking both the endothelial and the VSMC ET_B receptor does not result in additional inhibition of ET-induced O_2^- production. In accordance with these findings we also noted an increase in DHE signal coming from the media which indicates that the ET-induced O_2^- production predominantly originates from vascular smooth muscle cells which have an abundance of ET_A receptors (Schiffrin, 2001). Interestingly, O_2^- generation may increase atherosclerosis by activating VSMC mitogenic signalling pathways (Vendrov et al., 2007). This finding together with our results indicates that blocking ET-induced O_2^- production may be one mechanism behind the anti-atherosclerotic effect of ET receptor blockade (Barton et al., 1998).

It is not clear which enzymatic source of O_2^- that causes ET-mediated O_2^- production and therefore we sought to study this further. Since inhibition of basal O_2^- has been studied in detail (Guzik et al., 2006) we sought to focus on the inhibition of ET-induced O_2^- production. In this study ET-induced O_2^- production is significantly inhibited only by Tiron — a superoxide scavenger — and DPI — an inhibitor of flavin dependent enzymes such as NADPH oxidase, xanthine oxidases and NOS. Specific inhibition of NOS, xanthine oxidases and mitochondrial enzymes did not inhibit ET-induced O_2^- indicating that it is unlikely that these enzyme systems contribute to ET-induced O_2^- production. Accordingly we conclude that NADPH oxidase is likely to have contributed substantially to the O_2^- production that was observed following incubation with ET-1. This is in

accordance with a previous report which demonstrated that ET-1 increases vascular O_2^- generation via NADPH oxidase in a model of low-renin hypertension (Li et al., 2003b). It is possible that this increase in O_2^- production is mainly due to a membrane-bound NADPH oxidase activity as suggested in a study on pig coronary arteries (Brandes et al., 1997). However, Ergul et al., 2005 could not detect any increase in NADPH oxidase-mediated O_2^- production following ET-1 incubation in HSV. This difference may be due to the fact that frozen and homogenized HSV instead of fresh IMA were used. Surprisingly apocynin did not inhibit O_2^- production in our study. Apocynin inhibits the release of O_2^- by NADPH oxidase by blocking p47 phox and its translocation to the membrane and therefore blocks the assembly of NADPH oxidase (Stolk et al., 1994). Apocynin is also a prodrug which requires metabolic activation (oxidation) by myeloperoxidases (MPO) in order to form active dimers (Wind et al., 2010) and there are conflicting data on whether endothelial cells and vascular smooth muscle cells can form these dimers and therefore also activate apocynin (Heumuller et al., 2008; Touyz, 2008). Since apocynin can act as a radical scavenger but also stimulate ROS generation as discussed by Heumuller and Touyz it seems imperative that any further study of whether NADPH oxidase is involved in ET-1 ROS generation in humans should include models that are not dependent on the inhibitor per se. With the current knowledge at hand and the vascular model chosen in this study we conclude that the absence of inhibition of O_2^- by apocynin in this vascular model does not necessarily exclude a role for NADPH oxidase in ET-1 mediated O_2^-.

In the human vasculature four isoforms of NADPH oxidase have been identified; nox 1 in endothelial cells (EC), nox 2 and 5 in EC and VSMC and nox 4 in all cell types. There is a significant increase in expression and activity of these nox subunits in the setting of vascular disease states such as diabetes, hypercholesterolemia and hypertension (Brandes et al., 2010; Leto et al., 2009; Montezano et al., 2011). NADPH oxidase-derived ROS results in negative modulation of vascular tone and stimulation of pro-inflammatory responses (Paravicini and Touyz, 2008). In our study we observed a significant increase in O_2^- production already after 45 min of ET-1 exposure suggesting a rapid stimulation of NADPH oxidase activity. To our knowledge there are no studies performed on human vessels describing the source of ET-mediated O_2^- production and a possible intracellular pathway in the same study. A previous report shows that ET-1 increases expression and activity of p47 phox in rat aortic rings via the ET_A receptor which would suggest that ET-1 is critically involved in the activation of NADPH oxidase (Romero et al., 2010). The signalling pathway suggested was sequential activation of protein kinase C (PKC), c-Src and ERK 1/2. Whether the ERK–MAPK pathway is involved in ET-induced O_2^- production is still unclear. Romero et al. (2010) could not see any acute (i.e. within 60 min) effect of ET-1 on O_2^- production but the effect described occurred after 2 h. It is therefore possible that in vessels from CAD patients, which may have upregulated pro-inflammatory activity, a different pattern of stimulation and activation of NADPH oxidase occurs.

The following limitations were identified; first the study cohort should ideally include healthy controls not only because of difference in vascular pathophysiology but also due to ongoing medication such as statins, ACE inhibitors and beta blockers which will have specific effects on O_2^- generation among patients. The finding that ET receptor blockade reduced O_2^- production in vessels from patients included in the present study may be of clinical importance since it demonstrates pathophysiological effects of ET-1 on top of standard treatment in CAD. Second, the ET-mediated O_2^- production was measured in vessels ex-vivo and the present findings cannot be extrapolated to in vivo conditions. However, it is of interest that ET-1 induces endothelial dysfunction also in vivo via a mechanism that is related to oxidative processes (Böhm et al., 2007). Third, the small amount of tissue limits the possibility for multiple observations in each patient. However, each observation was from paired samples from the same patient and the study cohort was relatively large.

Conclusions

We conclude that ET-1 increases O_2^- production especially in human arteries and less so in veins from patients with CAD via a receptor-dependent pathway involving NADPH oxidase. Production of O_2^- may contribute to negative effects of ET-1 on vascular function such as impairment of endothelium-dependent vasodilatation and pro-inflammatory effects. Beneficial effects of ET receptor blockade in patients with CAD may be related to reduced production of O_2^-.

Conflict of interest statement

None.

Acknowledgements

We gratefully acknowledge the skilled help in harvesting vessels from the cardiothoracic surgeons Ravi Sayeed, Chandi Ratnatunga, Ravi Pillai and David Taggart at John Radcliffe Hospital, Oxford, UK. We also gratefully acknowledge Dr Tim van Assche and research assistant Jonathan Diesch in contributing to sample handling. Furthermore, we acknowledge the help and fruitful discussions with Dr Nick Alp and laboratory technician Ashley Hale. This study was funded by the Swedish Heart–Lung foundation, the Swedish Medical Research Council (10857), the Erik and Edith Fernström foundation, the Stockholm County Council (ALF), Karolinska Institutet/Stockholm County Council Strategic Cardiovascular Programme, and the King Gustav V and Queen Victoria Foundation. Furthermore, laboratory expenses were funded through grants for KM Channon.

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