Microvascular Research 78 (2009) 45–50

Contents lists available at ScienceDirect

Microvascular Research

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

Regular Article

Dependence of cathepsin L-induced coronary endothelial dysfunction upon activation of NAD(P)H oxidase

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ARTICLE INFO

Article history:
Received 3 February 2009
Revised 23 March 2009
Accepted 24 March 2009
Available online 1 April 2009

Keywords:
Proteinase
Angiogenesis inhibitor
Coronary circulation
Endothelium
Free radicals

ABSTRACT

Cathepsin L is a cysteine protease that can generate endogenous endostatin in vascular and epithelial basement membranes and importantly participates in a variety of pathophysiological processes. The present study was designed to determine whether this cathepsin L-derived endogenous endostatin alters endothelium-dependent vasodilator responses in coronary arteries via NAD(P)H oxidase activation. In isolated and perfused small bovine coronary arteries, administration of cathepsin L (200 ng/ml) markedly attenuated endothelium-dependent vasodilator responses to bradykinin or A23187 by 56.16 ± 9.58% and 68.95 ± 10.32%, respectively. This inhibitory effect of cathepsin L on endothelium-dependent vasodilator responses could be significantly reversed by pre-incubation of the arteries with O_2^- scavenger, Tiron, or neutralizing anti-endostatin antibody. By fluorescent ELISA assay, cathepsin L dose-dependently increased endostatin production in coronary arteries. *In situ* high-speed dual wavelength switching fluorescent microscopic imaging showed that cathepsin L decreased bradykinin- and A23187-induced NO levels in the intact endothelium, but it had no effect on Ca^{2+} response to these vasodilators. This cathepsin L-induced reduction of NO was restored by the pretreatment of an anti-endostatin antibody. Electron spin resonance (ESR) analysis demonstrated that cathepsin L increased O_2^- production which could be markedly attenuated by the NAD(P)H oxidase inhibitors, apocynin or anti-endostatin antibody. It is concluded that endostatin could be endogenously produced in coronary arteries when cathepsin L is increased and that this cathepsin L-derived endostatin, if excessive, may result in endothelial dysfunction through enhanced production of O_2^- due to NAD(P)H oxidase activation.

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Introduction

Cathepsins, known as cysteine proteases, have been found in many types of cells from plants to animals, which share the same features of endosomal targeting motifs, namely, acidic pH optima and autocatalytic activation. Physiologically, cathepsins have a vital role in a number of events, such as antigen presentation (Nakagawa et al., 1998), bone absorption (Saftig et al., 1998), biosynthesis of peptide neurotransmitter and hormones (Hook et al., 2004). Under pathological conditions, these elastinolytic cathepsins are highly expressed as observed in atherosclerosis, chronic obstructive pulmonary disease, and malignant tumors (Cimerman et al., 2001; Kitamoto et al., 2007; Liotta et al., 1991). Among these cathepsins, cathepsin L is considered as a potent isoform contributing to the process of atherogenesis. Although the underlying mechanism is not fully understood, some observations have suggested that cathepsin L participates in atherosclerosis by degradation of elastin and collagen and consequent regulation of blood-borne leukocyte transmigration and lesion progression. In particular, loss of collagen XVIII under action of cathepsin L was found to importantly enhance neovascularization and vascular permeability in atherosclerosis (Moulton et al., 2004).

More recently, collagen XVIII as the core protein of a heparin sulfate proteoglycan in vascular and epithelial base (Felbor et al., 2000; Oh et al., 1994; Sasaki et al., 1998) has been shown to contain a domain as the substrate of cathepsin L. It was demonstrated that activation of cathepsin L causes the cleavage of peptide bonds within the protease-sensitive hinge region of the NC1 domain of collagen XVIII, leading to the release of endostatin (Felbor et al., 2000; Oh et al., 1994), a ~20-kDa proteolytic fragment (O'Reilly et al., 1997). To date, endostatin has been reported as one of the most potent endothelial cell-specific inhibitors of angiogenesis and tumor growth *in vivo* (Boehm et al., 1997; Dhanabal et al., 1999b; O'Reilly et al., 1997). This collagen XVIII-derived antiangiogenetic factor also has been demonstrated to inhibit endothelial cell growth or induce apoptosis *in vitro* (Dhanabal et al., 1999a; O'Reilly et al., 1997). Recent studies in our laboratory and by others have demonstrated that exogenous endostatin reduces nitric oxide (NO) production in intact coronary arterial endothelium or in cultured umbilical vein endothelial cells (Urbich et al., 2002) which may induce endothelial dysfunction preceding structural or morphological damages such as apoptotic or necrotic

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0026-2862/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.mvr.2009.03.006

injury of these cells. However, so far there is no direct evidence that endogenously produced endostatin does produce endothelial dysfunction.

In the present study, we first tested whether endostatin could be produced from coronary arterial preparation via cathepsin L and cathepsin L-derived endostatin reduces endothelial NO production in intact coronary artery and thereby impairs endothelium-dependent vasodilation in coronary arteries. We then further explored a Ca^{2+}-independent and superoxide ($O_2^{\cdot -}$)-associated mechanism reducing NO in coronary endothelial cells. Finally, we examined the contribution of NAD(P)H oxidase to the effects of cathepsin L-derived endostatin in inducing $O_2^{\cdot -}$ production and NO–$O_2^{\cdot -}$ interactions.

Materials and methods

Dissection of small coronary arteries

Fresh bovine hearts were obtained from a local abattoir. Small intramural coronary arteries from the branches of left anterior descending artery were carefully dissected and placed in the ice-cold physiological saline solution (PSS) containing the following composition (in mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 1.6; $MgSO_4$, 1.17; NaH_2PO_4, 1.18; $NaHCO_3$, 24; EDTA, 0.026; and glucose, 5.5, pH 7.4. For microperfusion experiments, the artery segments (250 ± 50 μm in lumen diameter; 5–8 mm in length) were carefully cleaned off of fat and connective tissues under a dissection microscope and placed in cold PSS until cannulation. For fluorescence imaging experiments, small coronary arteries (300–500 μm in lumen diameter) were prepared in a similar way and kept in ice-cold modified Hanks' buffered salt solution containing (in mM) 137 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 3 Na_2HPO_4, 0.4 KH_2PO_4, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 0.8 $MgSO_4$, 10 glucose, 10 HEPES, pH 7.4 (Li et al., 1999; Yi et al., 2002; Zhang et al., 2006b).

ELISA immunofluorescence analysis of endostatin production in coronary arteries by cathepsin L

The left anterior descending artery from fresh bovine heart was dissected and homogenized in ice-cold HEPES buffer (pH 7.4) containing (in mM) 20 HEPES, 1 EDTA, 255 sucrose with complete protease inhibitor cocktail (Roche Diagnostics). After centrifugation of the homogenate at 6000 *g* for 5 min at 4 °C, the supernatant containing the membrane protein and cytosolic components was collected and termed as homogenate. 100 μg homogenate protein was incubated with 100 ng or 200 ng cathepsin L for 1 h. Endostatin level was determined using Quantikine endostatin immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Vascular reactivity in in vitro perfused small coronary arteries

Dissected arteries were transferred to a water-jacketed perfusion chamber and cannulated with two glass micropipettes at their *in situ* length as described previously (Campbell et al., 2002; Geiger et al., 2000; Zhang et al., 2005). The outflow cannula was clamped and the arteries were pressurized to 60 mm Hg and equilibrated in PSS at 37 °C. PSS in the bath was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 throughout the experiment. After a 1-hour equilibration period, the arteries were precontracted with a thromboxane A_2 analog, U-46619 (2–20 nM) until a ~50% of decrease in resting diameter was reached. Once steady-state contraction was obtained, cumulative dose–response curves to the endothelium-dependent vasodilator bradykinin (BK, 10^{-10} to 10^{-6} mol/l) or A23187 (10^{-9} to 10^{-5} mol/l) were determined by measuring changes in internal diameter. All drugs were added into the bath solution unless otherwise indicated. The vasodilator response was expressed as the percent relaxation of U-46619-induced precontraction based on changes in arterial internal diameter. The arteries were excluded from statistical analysis if the contractile response to U46619 was <40% or dilator response to bradykinin <80%. Internal arterial diameter was measured with a video system composed of a stereomicroscope (Leica MZ8), a charge-coupled device camera (KP-MI AU, Hitachi), a video monitor (VM-1220U, Hitachi), a video measuring apparatus (VIA-170, Boeckeler Instrument), and a video printer (UP890 MD, Sony). The arterial images were recorded continuously with a videocassette recorder (M-674, Toshiba). All of the drugs were added into the bath solution unless otherwise indicated. The vasodilator response to bradykinin and A23187 was expressed as the percent relaxation of U46619-induced precontraction based on changes in the ID.

Electromagnetic spin resonance (ESR) spectrometric detection of $O_2^{\cdot -}$

Primarily cultured bovine coronary artery endothelial cells were gently collected and suspended in modified Krebs-HEPES buffer containing deferoxamine (100 μM; metal chelator). Approximately 1×10^{-6} cells were then incubated with cathepsin L (200 ng/ml) for 1 h or endostatin (100 nM) for 30 min in the presence of endostatin antibody (10 μg/ml) or apocynin (100 μM), an NAD(P)H oxidase inhibitor, then mixed with 1 mM of the O_2^- specific spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH). The cell mixture was subsequently loaded in glass capillaries and immediately kinetically analyzed for O_2^- production at each minute for 10 min (Noxygen Science Transfer & Diagnostics GmbH). The ESR settings were as follows: biofield, 3350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; 4096 points of resolution; receiver gain, 100; and kinetic time, 10 min (Jin et al., 2008b; Zhang et al., 2008).

Fluorescence imaging analysis of NO levels and $[Ca^{2+}]_i$ in the intact endothelium of bovine coronary arteries

Simultaneous recording of NO level and $[Ca^{2+}]_i$ in the intact endothelium of coronary was performed with a protocol developed currently in our laboratory, in which fura 2 (Zhang et al., 2004) was used as the indicator for $[Ca^{2+}]_i$ and 4,5-diaminofluorescein (DAF-2) as the probe for intracellular NO levels (Kojima et al., 1998; Yi et al., 2002; Zhang et al., 2005). In brief, small bovine coronary artery (300–500 μm in diameter) with the lumen open along its longitudinal axis was loaded with DAF-2 diacetate (10 μM) and fura 2-AM (10 μM) in Hanks buffer containing (in mM), 137 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 3 Na_2HPO_4, 0.4 KH_2PO_4, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 0.8 $MgSO_4$, 10 glucose, and 10 HEPES; pH 7.4 for 40 min at room temperature, then transferred into recording chamber with the vessel lumen side facing a ×20 phase/fluor objective (Nikon Diaphot) for individual endothelial cells visualization. The dynamic changes in $[Ca^{2+}]_i$ and NO level within the cells were analyzed with the excitation wavelengths of 340 ± 10 or 380 ± 10 (both for fura 2), and 480 ± 20 (for DAF-2) nm provided by a high-speed wavelength switcher (Lambda DG-4; Sutter, Novato, CA), and emission wavelength of 510 ± 20 nm for fura 2 or 535 ± 25 nm for DAF-2 by a high-speed rotating filter wheel (Lambda 10–2; Sutter). The fluorescence images were captured by a digital camera (SPOT RT Monochrome; Diagnostic Instruments). A metafluor imaging and analysis software (Universal Imaging) was used to acquire, digitize, and store the images and for off-line processing and statistical analysis.

The fluorescence ratio of excitation at 340 nm to that at 380 nm (F340/F380) was determined after background subtraction, and $[Ca^{2+}]_i$ was calculated by using the following equation: $[Ca^{2+}]_i = Kd[(R - Rmin)/(Rmax - R)]$, where Kd for the fura 2-Ca^{2+} complex is 224 nM; R is the fluorescence ratio (F340/F380); Rmax and Rmin are the maximal and minimal fluorescence ratios measured by addition of 10 μM of Ca^{2+} ionophore ionomycin to Ca^{2+}-replete solution (2.5 mM $CaCl_2$) and Ca^{2+}-free solution (5 mM EGTA), respectively; and R is the