DEPENDENCE OF CATHEPSIN L –INDUCED CORONARY ENDOTHELIAL DYSFUNCTION UPON ACTIVATION OF NAD(P)H OXIDASE

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ABSTRACT

Cathepsin L can generate endogenous endostatin in vascular and epithelial basement. The present study was designed to determine whether and how this cathepsin L–derived endogenous endostatin alters endothelium-dependent vasodilator responses in coronary arteries. In isolated and perfused small bovine coronary arteries, 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 could be significantly reversed by pre-treatment with O_2^- scavenger, tiron, or anti-endostatin antibody. ELISA assay revealed that cathepsin L dose dependently increased endostatin production in coronary arteries. In situ fluorescent microscopic imaging showed that cathepsin L decreased bradykinin- and A23187-induced NO production in the intact endothelium, but with no effect on Ca^{2+} response. This cathepsin L-induced reduction of NO was restored by the pretreatment of an anti-endostatin antibody and tiron. Electron spin resonance (ESR) analysis demonstrated that cathepsin L increased O_2^- production, which could be markedly attenuated by apocynin or anti-endostatin antibody. It is concluded that endostatin could be endogenously produced in coronary arteries, which may result in endothelial dysfunction by NADPH oxidase activation (Supported by NIH grants HL57244, HL075316, and DK54927).

METHODS

ELISA immunofluorescence analysis of endostatin production in coronary arteries by cathepsin L. The left anterior descending artery from fresh bovine hearts were 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 6,000 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.

Electromagnetic Spin Resonance (ESR) Spectrometric Detection of O_2^-. 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, 3,350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; 4,096 points of resolution; receiver gain, 100; and kinetic time, 10 min.

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 currently developed in our laboratory, in which fura 2 was used as the indicator for $[Ca^{2+}]_i$ and 4,5-diaminofluorescein (DAF-2) as the probe for intracellular NO levels (*Li et al. Am J Physiol Heart Circ Physiol. 2002 ;283(6):H2725-32*).

BACKGROUND

1. Cathepsin L is a cysteine protease, which has been demonstrated to proteolyze collagen XVIII in vascular and epithelial basement membranes to generate endogenous endostatin and importantly participates in a variety of pathophysiological processes.

2. 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, which may induce endothelial dysfunction.(*Li Pl et al Am J Physiol Heart Circ Physiol.2005; 288: H686-694, Urbich C et al. Faseb J.2002; 16: 706-708*).

3. The present study was designed to determine whether cathepsin L–derived endogenous endostatin produces endothelial dysfunction and to explore the role of NAD(P)H oxidase in the reduction of NO bioavailability in coronary arteries.

RESULTS

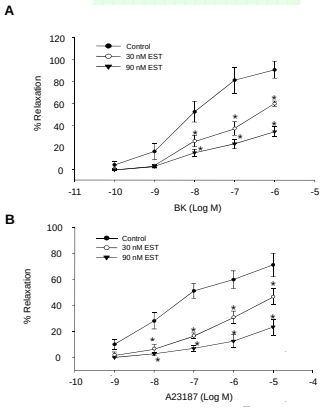

Fig. 1. Dose-dependent inhibition effects of endostatin on concentration-dependent vasodilator response in freshly isolated and pressurized small bovine coronary arteries. A: Effects of endostatin (30 and 90 nM) on vasodilator responses to BK with maximal inhibition of 34.14 ± 5.09% and 62.36 ± 6.16%, respectively. B: Effects of endostatin (30 and 90 nM) on vasodilator responses to A23187 with maximal inhibition of 34.28 ± 7.77% and 67.20 ± 6.30% in 30 and 90 nM endostatin group, respectively. These results indicate that endostatin can induce endothelium dysfunction.

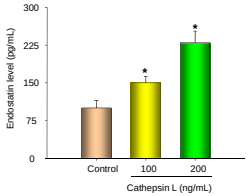

Fig. 2. Cathepsin L concentration-dependently increases endostatin production in bovine coronary artery homogenates by ELISA assay. Summarized data showing tissue level of endostatin markedly increased by about 50% and 130% in 100 and 200 ng/ml cathepsin L-treated group, respectively, compared with control. These results suggest that endostatin could be endogenously produced.

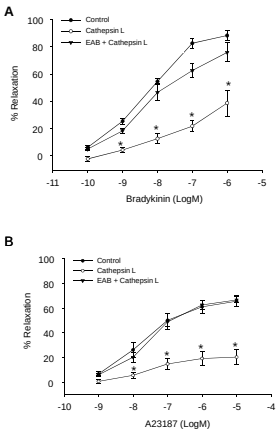

Fig. 3. Effects of anti-endostatin antibody (EAB) on cathepsin L-induced vasodilation damage in small bovine coronary arteries. A: Cathepsin L alone significantly decreases BK induced vasodilation, pretreated with EAB (10 µg/ml) almost completely prevents cathepsin L (200 ng/ml)-induced vasodilation damage to BK. B: Similarly, Cathepsin L alone significantly decreases A23187-induced vasodilation, whereas pretreatment with EAB (10 µg/ml) abolished cathepsin L (200ng/ml)-induced vasodilation damage to A23187. These results indicate that cathepsin L-induced endothelium dysfunction is mediated by endostatin.

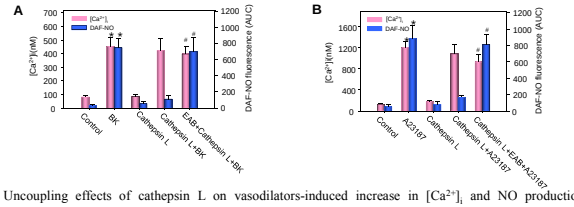

Fig. 4. Uncoupling effects of cathepsin L on vasodilators-induced increase in $[Ca^{2+}]_i$ and NO production [DAF-Fluorescence, area under the curve (AUC) in 30 min] in intact endothelium of small coronary. BK or A23187 alone significantly increased $[Ca^{2+}]_i$ and DAF fluorescence. Pretreated with cathepsin L, either BK or A23187 no longer increased NO but still stimulated an increase in $[Ca^{2+}]_i$. However, in the presence of both anti-endostatin antibody (EAB) (10 µg/ml) and cathepsin L (200 ng/ml), BK or A23187 -induced NO production was restored to the level when the arteries were stimulated by BK or A23187 alone. A: BK treatment results, B: A23187 treatment results.

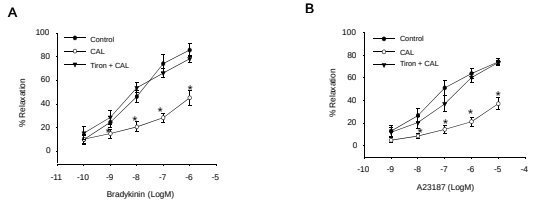

Fig. 5. Effects of SOD mimetic tiron on cathepsin L-induced impairment of the vasodilator responses. A: In Tiron + CAL (Cathepsin L) group, pretreated with Tiron (1 mM) almost completely prevented CAL-induced vasodilation damages to BK, compared with CAL alone. B: Similarly, in Tiron + CAL group, pretreated with Tiron (1 mM) almost fully recover CAL-induced vasodilation damages to A23187.

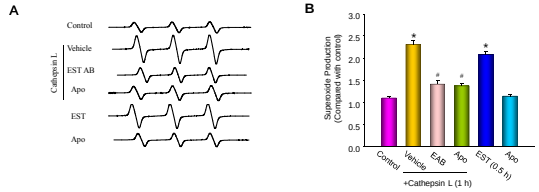

Fig. 6. Electron spin resonance (ESR) spectrometric analysis of O_2^- production associated with NAD(P)H oxidase in bovine coronary arterial endothelial cells stimulated by endostatin and cathepsin L. A: representative ESR spectrographs of O_2^- trapped by 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) with NAD(P)H as substrate. B: Summarized data showing either cathepsin L or endostatin (EST) markedly increase O_2^- production. Preatreatment of endothelial cells with endostatin antibody (EAB, 10 µg/ml) or NAD(P)H oxidase inhibitor, apocynin (Apo, 100 µM), significantly attenuated cathepsin L-induced O_2^- production, whereas apocynin alone had no effects on basal O_2^- production.

CONCLUSION

1. ***The present study demonstrates that cathepsin L promotes endogenous generation of endostatin and attenuates the endothelium-dependent vasodilator response in bovine coronary arteries.***

2. ***The endogenously generated endostatin by cathepsin L could activate NAD(P)H oxidase and increase O_2^- generation, resulting in decreased NO bioavailability in the endothelium, and leading to endothelial dysfunction.***