Published in final edited form as:

Vet Immunol Immunopathol. 2010 July ; 136(1-2): 127–132. doi:10.1016/j.vetimm.2010.02.006.

Endothelin-1 production by the canine macrophage cell line DH82: Enhanced production in response to microbial challenge

Jeffrey N. Divino, Kashmira S. Chawla, Christina M. da Silva, Ashley M. Bjorge, and Andrew Brittingham*

Department of Microbiology and Immunology, Des Moines University, College of Osteopathic Medicine, Des Moines, Iowa 50312

Abstract

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide which plays an important role in regulating mammalian cardiovascular development and homeostasis. Originally identified as a factor released by vascular endothelial cells, ET-1 is now recognized as a product of numerous cells and tissues with demonstrated involvement in an array of physiological and pathological processes. An area of great interest is the production of ET-1 by mononuclear cells (monocytes and macrophages) and its role in inflammation. We report that the canine macrophage cell line, DH82, constitutively secretes both ET-1 and its biologically inactive precursor big ET-1. The production of both peptides was increased following stimulation with lipopolysaccharide (endotoxin) from gram-negative bacteria. ET-1 production was also increased in response to stimulation with intact and viable gram-positive and gram-negative bacteria. In addition to producing ET-1, DH82 cells express transcripts encoding two receptors for the ET-1 peptide (ET_A and ET_B receptors) and an enzyme involved in the conversion of big ET-1 to ET-1. The constitutive secretion of ET-1 and the expression of ET_A and ET_B receptors may be related to the malignant origin of this cell line. Our results are the first report of ET-1 production by a canine cell line and provide the basis for further investigation into the role of ET-1 during infection and inflammation.

Keywords

macrophage; endothelin-1; lipopolysaccharide

1. Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide, first purified from the conditioned media of cultured porcine endothelial cells (Yanagisawa et al., 1988). The endothelin system, which includes at least three related peptides, at least two endothelin specific receptors and several enzymes involved in peptide processing and activation has been identified in most mammals, as well as several fish, amphibian and invertebrate species (Miller et al., 2000;

© 2010 Elsevier B.V. All rights reserved.

*Corresponding author: Andrew Brittingham, PhD, Department of Microbiology and Immunology, Des Moines University, College of Osteopathic Medicine, 3200 Grand Ave., Des Moines, Iowa 50312, Telephone: 515-571-1508, FAX: 515-271-1543, Andrew.Brittingham@dmu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Zhang et al., 2001; Macours et al., 2004; Masaki. 2004; Quan et al., 2004; Wang et al., 2006).

ET-1 is initially synthesized as a 212 amino acid precursor known as preproendothelin-1 (ppET-1). This molecule is quickly processed into a 39 amino acid biologically inactive intermediate known as big ET-1. Big ET-1 is subsequently processed into the 21 amino acid biologically active ET-1 through the activity of a group of metalloproteases known as endothelin-converting enzymes. The biological effects of ET-1 are mediated by the ligation of at least two G-protein coupled receptors, the ET_A and ET_B receptors.

Since its identification, most studies have focused on the role of ET-1 in cardiovascular physiology and pathophysiology. ET-1 has been demonstrated to play a central role in both cardiovascular homeostasis and cardiovascular embryogenesis (Kurihara et al., 1994; Clouthier et al., 1998; Haynes and Webb, 1998), with excessive or aberrant ET-1 production being associated with numerous cardiovascular pathologies including chronic heart failure, hypertension, atherosclerosis, and pulmonary hypertension (Best and Lerman, 2000; Teder and Noble, 2000).

In addition to its vasoconstrictive properties, ET-1 acts as a mitogen for vascular-smooth muscle cells, modulates the expression of adhesion molecules on endothelial cells, and is a chemoattractant and modulator of immune cell function (Battistini et al., 1996; Wanecek et al., 2000; Kedzierski and Yanagisawa, 2001).

Elevated plasma and tissue levels of ET-1 have been noted in numerous human and veterinary clinical pathologies, including cardiopulmonary diseases (pulmonary hypertension, congestive heart failure, cardiomyopathy), as well as systemic and localized inflammatory conditions such as bronchial asthma, arthritis, inflammatory bowel disease, atherosclerosis, and sepsis (Nakamura et al., 1991; Weitzberg et al., 1991; Best and Lerman, 2000; Wanecek et al., 2000; Prosek et al., 2004; Tessier-Vetzel et al., 2006; O'Sullivan et al., 2007). Recent studies have reported elevated levels of ET-1 in the plasma of dogs suffering from heartworm disease (dirofilariasis) and in the eye of glaucomatous dogs (Uchida and Saida, 2005; Kallberg et al., 2007).

While historically viewed as an endothelium derived regulator of vascular tone, ET-1 is now known to be produced by a wide variety of cells and tissues under normal and pathological conditions. Vascular smooth muscle cells, monocytes, macrophages, (Liu et al., 1997) mast cells, cardiomyocytes, and epithelial cells have all been demonstrated to produce ET-1 (Wanecek et al., 2000; Kedzierski and Yanagisawa, 2001).

The identification of macrophages as a source of ET-1 during the development of atherosclerosis and septic shock has been widely reported in human patients and experimental animal models (Zeiber et al., 1995; McMillen and Cunningham, 1996; Ebihara et al., 1998). Human, rat, mouse, and guinea pig mononuclear phagocytes (monocytes / macrophages) have all been demonstrated to produce ET-1 in response to a variety of stimuli (Ehrenreich et al., 1990; Liu et al., 1997; Xu and Zhong, 1997; Wahl et al., 2005).

Here we report our characterization of ET-1 production by the canine macrophage cell line DH82. We demonstrate that DH82 cells constitutively secrete ET-1, but increase production in response to stimulation with endotoxin (LPS) or intact gram-positive or gram-negative bacteria. Additionally, these cells express transcripts (mRNAs) encoding all components of the endothelin axis: preproendothelin-1, endothelin-converting enzyme, and the endothelin receptors making DH82 cells a valuable tool for analyzing the endothelin system of mononuclear phagocytes.

2. Materials and methods

2.1. Cell culture, microorganisms, and reagents

The canine macrophage cell line DH82 (CRL-10389) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 500 I.U. / ml penicillin G, and 500 µg / ml streptomycin. Cells were grown at 37°C in 5% CO₂ and passaged semi-weekly. Twenty-four hours before use, cells were removed from culture dishes by treatment with 5 mM EDTA in PBS, washed, and resuspended in cell culture media. Cells were plated at a density of 5×10⁵ cells / ml into 24-well plates (0.5 ml / well) or 60 mm tissue culture plates (3 ml / plate).

Escherichia coli (ATCC#25922) and *Staphylococcus aureus* (ATCC#29213), were purchased from ATCC. Bacteria were grown overnight at 37°C in Brain Heart Infusion Broth (Difco, Detroit, MI). Prior to being used in macrophage stimulation assays, all microorganisms were washed extensively in Hank's balanced salt solution (HBSS; Mediatech).

Bacterial lipopolysaccharide (LPS; *E. coli* 0127:B8) and SM-19712 were both purchased from Sigma (St. Louis, MO) and constituted in Dulbecco's phosphate buffered saline (PBS; Mediatech).

For DH82 stimulation assays, intact and viable microorganisms were added to DH82 monolayers to achieve a multiplicity of infection of approximately 10 to 1. LPS was added to achieve the final concentrations indicated. At the indicated times, culture supernatants were collected and frozen (−80°C) for later analysis. In some assays, the endothelin converting enzyme inhibitor, SM-19712 was added 20 minutes prior to DH82 stimulation.

2.2. Analysis of ET-1 and big ET-1 peptide levels

The amount of ET-1 or big ET-1 peptide present in culture supernatants was determined using the endothelin-1 and rat big endothelin-1 EIA kits from Assay Designs, Inc. (Ann Arbor, MI). The canine ET-1 peptide (21 amino acids) is 100% identical to that of ET-1 in most species, including human, mouse, and rat. The canine big ET-1 peptide (39 amino acids) is 95% identical to rat big ET-1 (Kimura et al., 1989; Uchide and Saida, 2005).

2.3. Detection of mRNAs encoding components of the endothelin axis

RNA was isolated from washed DH82 monolayers using the Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, MN). cDNA was synthesized from 1 µg of total RNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI). Residual genomic DNA was removed from RNA samples with a TURBO DNA-free Kit (Ambion, Austin, TX). PCRs were performed using GoTaq Green Master Mix (Promega, Madison, WI) and the primers described in Table 1 to amplify cDNAs encoding preproendothelin-1, endothelin converting enzyme-1, endothelin receptor A, endothelin receptor B, and the housekeeping gene GAPDH. Amplification parameters consisted of polymerase activation at 95°C for 90 sec, followed by 30 cycles comprised of 95°C separation (for 30 sec), 60°C anneal (45 sec), and 72°C extension (45 sec) steps, and a final 72°C extension (10 min). PCR products were separated by electrophoresis on agarose gels containing ethidium bromide. Volumes of cDNA input were adjusted, if necessary, relative to intensities of GAPDH bands. Gels were photographed using a FOTO/Analyst Investigator gel documentation system (FOTODYNE, Hartland, WI).

2.4. Statistical analysis

Data were analyzed using Student's *t*-tests or analysis of variance (ANOVA). ANOVAs were followed by Dunnett's tests to compare each treatment group to the control group. Analysis was performed using SPSS (SPSS Inc., Chicago, IL) and results where $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Constitutive and LPS-induced ET-1 production

Human and murine mononuclear cells produce ET-1 in response to stimulation with bacterial lipopolysaccharide (LPS) (Ehrenreich et al., 1990; Wahl et al., 2005). In fact, in the absence of stimulation these cell populations produce little or no ET-1 (Ehrenreich et al., 1990; Wahl et al., 2005), a property unique from those observations made in other cell types such as endothelial cells (Yanagisawa et al., 1988). To determine the ability of DH82 cells to produce ET-1, cells were cultured in the absence or presence of 100 ng / ml of LPS for periods of time ranging from 2 to 26 hours. At given time points culture supernatants were collected and assayed for the presence of ET-1 peptide.

Contrary to the observations made with primary human and murine mononuclear cells, DH82 cells constitutively secrete ET-1, with the concentration of ET-1 continuing to increase throughout the incubation period (Fig. 1A). Similar to observations made in human and murine cell populations, LPS stimulation resulted in a rapid and significant increase in the concentration of ET-1 detected in culture supernatants. In all assays, a significant increase in ET-1 production was apparent within 4 hours of LPS stimulation, and continued throughout the incubation period (P 's < 0.01).

To further analyze LPS induced ET-1 production by DH82 cells, cells were cultured in the absence of LPS, or in the presence of increasing concentration of endotoxin (0.001 to 1 ug / ml). Increased ET-1 production was observed in all assays using as little as 10 ng / ml of LPS (P 's < 0.01), with maximum induction achieved at a concentration of 100 ng / ml (Fig. 1B). These data demonstrate that LPS stimulation increases DH82 ET-1 production in a time and dose dependent fashion.

The constitutive expression of ET-1 in the absence of stimulation has not previously been reported for any primary mononuclear cell populations or cell lines. Studies in our lab using murine macrophage-like cell lines (J774, RAW264) have only been able to demonstrate significant endothelin production following cell stimulation with LPS or other agents (data not shown). Furthermore, we have been unable to demonstrate significant endothelin production by human pro-monocytic (THP-1, U937) or pro-myelocytic (HL-60) cell lines under any conditions (data not shown).

DH82 cells are derived from neoplastic progenitor cells isolated from the bone marrow collected from a case of canine malignant histiocytosis (Wellman et al., 1988). The neoplastic origin of DH82 cells may be related to its constitutive secretion of ET-1. ET-1 has been demonstrated to serve as an autocrine growth factor for numerous tumor cells and cell lines, including those isolated from breast, prostate, ovarian, colon and skin cancers (Grant et al., 2003; Bagnato and Natali, 2004). However, preliminary studies in our lab have shown that treatment of DH82 cells with selective endothelin receptor A (BQ-123) and B (BQ-788) antagonist had no effect on cell growth (data not shown).

3.2. ET-1 production in response to microbial challenge

Elevated levels of plasma ET-1 have been reported in both clinical cases and experimental animal models of both gram-negative and gram-positive sepsis (Battistini et al., 1996; Wanecek et al., 2000). In addition to the purified endotoxin component of gram-negative bacteria, murine macrophages produce ET-1 in response to intact and viable gram-positive as well as gram-negative bacteria (Wahl et al., 2005). To measure the response of DH82 cells to bacterial stimulation, cells were cultured in the absence or presence of intact and viable gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*). As illustrated in Figure 2, the exposure of DH82 cells to either gram-positive or gram-negative bacteria resulted in the increased production of ET-1 by these cells (P 's ≤ 0.004). This findings support the notion that ET-1 is part of the characteristic macrophage response to microbial challenge, suggesting a role for this peptide in orchestrating the immune response to infection.

3.3. Secretion of big ET-1

A critical step in the biosynthesis of ET-1 is the conversion of the biologically inactive pro-form of ET-1 (big ET-1) to mature ET-1. This is accomplished primarily through the activity of endothelin-converting enzyme-1 (ECE-1). In pathological conditions increased plasma and tissue levels of ET-1 as well as big ET-1 have been measured (Ishibashi et al., 1991; Rubens et al., 2001; Ihling et al., 2004; Masson et al., 2006; Yildirim et al., 2008), and in addition to ET-1, numerous cell types have been reported to secrete big ET-1 (Emori et al., 1989; Sawamura et al., 1989; Odoux et al., 1997). To assess the ability of DH82 cells to secrete big ET-1 in addition to ET-1, cells were cultured in the presence or absence of LPS, and with the addition of increasing concentrations of the endothelin-converting enzyme inhibitor SM-19712.

As demonstrated in Figure 3, and similar to what we observed with ET-1 (Fig. 3A), DH82 cells constitutively secrete big ET-1 and increase its production in response to LPS stimulation (Fig. 3B; $P < 0.001$). When peptide concentration (pg / ml) is converted to molarity, based on the individual molecular weight of each peptide, in the presence or absence of LPS, only 53% and 35% respectively, of the total endothelin-1 (big ET-1 and ET-1) present in culture supernatants is in the ET-1 form. The inclusion of an endothelin-converting enzyme inhibitor decreased the amount of mature ET-1 and increased the amount of big ET-1 detected in the culture media (Fig. 3; P 's ≤ 0.001). These data demonstrate that the amount of big ET-1, and therefore preproET-1, synthesized by DH82 cells, both constitutively and in response to LPS, exceeds the capacity of endothelin converting enzymes expressed by these cells.

3.4. Expression of the endothelin axis

Studies in human, mouse, and rat mononuclear cell types have reported that in addition to secreting ET-1, these cell types express receptors for ET-1 (ET_A and ET_B receptors) (Sakurai-Yamashita et al., 1997; Shimada et al., 1998; Haug et al., 2001). Some studies have reported that the ligation of endothelin receptors on macrophages can result in increased cytokine production and the increased production of reactive oxygen species (Haller et al., 1991; Kojima et al., 1994; Ruetten and Thiemermann, 1997; Speciale et al., 1998). To fully characterize the expression of the endothelin axis by DH82 cells, we performed reverse-transcriptase PCR (RT-PCR) on cDNAs derived from DH82 cells cultured in the absence or presence of LPS. As demonstrated in Figure 4., and similar to mononuclear cell types in other species, DH82 cells constitutively express transcripts encoding all essential components of the endothelin axis (preproET-1, ECE-1, and ET_A and ET_B receptors).

The endothelin axis is garnering increased attention as a potential target for therapies designed to treat cardiovascular disease, cancers, and inflammatory pathologies. The import role of macrophages as a source of ET-1 during many of these disease states is being increasingly

recognized. Our results provide a framework for the continued analysis of the induction and regulation of ET-1 production by macrophages, and may also provide a valuable tool to analyze the contribution of ET-1 to canine malignancies.

Acknowledgments

This work was supported in part by NIH R15HL081124 (AB).

References

- Bagnato A, Natali PG. Endothelin Receptors as Novel Targets in Tumor Therapy. *J. Transl. Med* 2004;2:16. [PubMed: 15165288]
- Battistini B, Forget MA, Laight D. Potential Roles for Endothelins in Systemic Inflammatory Response Syndrome with a Particular Relationship to Cytokines. *Shock* 1996;5:167–183. [PubMed: 8696980]
- Best PJ, Lerman A. Endothelin in Cardiovascular Disease: From Atherosclerosis to Heart Failure. *J. Cardiovasc. Pharmacol* 2000;35:S61–S63. [PubMed: 10976784]
- Clouthier DE, Hosoda K, Richardson JA, Williams SC, Yanagisawa H, Kuwaki T, Kumada M, Hammer RE, Yanagisawa M. Cranial and Cardiac Neural Crest Defects in Endothelin-A Receptor-Deficient Mice. *Development* 1998;125:813–824. [PubMed: 9449664]
- Ebihara I, Nakamura T, Shimada N, Shoji H, Koide H. Effect of Hemoperfusion with Polymyxin B-Immobilized Fiber on Plasma Endothelin-1 and Endothelin-1 mRNA in Monocytes from Patients with Sepsis. *Am. J. Kidney Dis* 1998;32:953–961. [PubMed: 9856510]
- Ehrenreich H, Anderson RW, Fox CH, Rieckmann P, Hoffman GS, Travis WD, Coligan JE, Kehrl JH, Fauci AS. Endothelins, Peptides with Potent Vasoactive Properties, are Produced by Human Macrophages. *J. Exp. Med* 1990;172:1741–1748. [PubMed: 1701822]
- Emori T, Hirata Y, Ohta K, Shichiri M, Shimokado K, Marumo F. Concomitant Secretion of Big Endothelin and its C-Terminal Fragment from Human and Bovine Endothelial Cells. *Biochem. Biophys. Res. Commun* 1989;162:217–223. [PubMed: 2665737]
- Grant K, Loizidou M, Taylor I. Endothelin-1: A Multifunctional Molecule in Cancer. *Br. J. Cancer* 2003;88:163–166. [PubMed: 12610497]
- Haller H, Schaberg T, Lindschau C, Lode H, Distler A. Endothelin Increases [Ca²⁺]_i, Protein Phosphorylation, and O₂^{•−} Production in Human Alveolar Macrophages. *Am. J. Physiol* 1991;261:L478–L484. [PubMed: 1662920]
- Haug C, Schmid-Kotsas A, Zorn U, Schuett S, Gross HJ, Gruenert A, Bachem MG. Endothelin-1 Synthesis and Endothelin B Receptor Expression in Human Coronary Artery Smooth Muscle Cells and Monocyte-Derived Macrophages is Up-Regulated by Low Density Lipoproteins. *J. Mol. Cell. Cardiol* 2001;33:1701–1712. [PubMed: 11549348]
- Haynes WG, Webb DJ. Endothelin as a Regulator of Cardiovascular Function in Health and Disease. *J. Hypertens* 1998;16:1081–1098. [PubMed: 9794709]
- Ihling C, Bohrmann B, Schaefer HE, Technau-Ihling K, Loeffler BM. Endothelin-1 and Endothelin Converting Enzyme-1 in Human Atherosclerosis--Novel Targets for Pharmacotherapy in Atherosclerosis. *Curr. Vasc. Pharmacol* 2004;2:249–258. [PubMed: 15320823]
- Ishibashi M, Saito K, Watanobe K, Uesugi S, Furue H, Yamaji T. Plasma Endothelin-1 Levels in Patients with Disseminated Intravascular Coagulation. *N. Engl. J. Med* 1991;324:1516–1517. [PubMed: 2023617]
- Kallberg ME, Brooks DE, Gelatt KN, Garcia-Sanchez GA, Szabo NJ, Lambrou GN. Endothelin-1, Nitric Oxide, and Glutamate in the Normal and Glaucomatous Dog Eye. *Vet. Ophthalmol* 2007;10 Suppl 1:46–52. [PubMed: 17973834]
- Kedzierski RM, Yanagisawa M. Endothelin System: The Double-Edged Sword in Health and Disease. *Annu. Rev. Pharmacol. Toxicol* 2001;41:851–876. [PubMed: 11264479]
- Kimura C, Itoh Y, Ohkubo S, Ogi K, Onda H, Fujino M. Cloning and Sequencing of a Canine Gene Segment Encoding Mature Endothelin. *Nucleic Acids Res* 1989;17:3290. [PubMed: 2657658]
- Kojima T, Hattori K, Fujiwara T, Hirata Y, Kobayashi Y. Maturation Changes in Superoxide Anion Production of Rabbit Alveolar Macrophages. *Life Sci* 1994;55:911–915. [PubMed: 8057752]

- Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, Oda H, Kuwaki T, Cao WH, Kamada N. Elevated Blood Pressure and Craniofacial Abnormalities in Mice Deficient in Endothelin-1. *Nature* 1994;368:703–710. [PubMed: 8152482]
- Liu B, Zhou J, Chen H, Wang D, Hu D, Wen Y, Xiao N. Expression and Cellular Location of Endothelin-1 mRNA in Rat Liver Following Endotoxemia. *Chin. Med. J. (Engl)* 1997;110:932–935. [PubMed: 9772405]
- Macours N, Poels J, Hens K, Francis C, Huybrechts R. Structure, Evolutionary Conservation, and Functions of Angiotensin- and Endothelin-Converting Enzymes. *Int. Rev. Cytol* 2004;239:47–97. [PubMed: 15464852]
- Masaki T. Historical Review: Endothelin. *Trends Pharmacol. Sci* 2004;25:219–224. [PubMed: 15063086]
- Masson S, Latini R, Anand IS, Barlera S, Judd D, Salio M, Perticone F, Perini G, Tognoni G, Cohn JN. Val-HeFT investigators. The Prognostic Value of Big Endothelin-1 in More than 2,300 Patients with Heart Failure Enrolled in the Valsartan Heart Failure Trial (Val-HeFT). *J. Card. Fail* 2006;12:375–380. [PubMed: 16762801]
- McMillen MA, Cunningham ME. Origin of Endothelin in Sepsis. *Crit. Care Med* 1996;24:721–722. [PubMed: 8612431]
- Miller CT, Schilling TF, Lee K, Parker J, Kimmel CB. Sucker Encodes a Zebrafish Endothelin-1 Required for Ventral Pharyngeal Arch Development. *Development* 2000;127:3815–3828. [PubMed: 10934026]
- Nakamura T, Kasai K, Sekiguchi Y, Banba N, Takahashi K, Emoto T, Hattori Y, Shimoda S. Elevation of Plasma Endothelin Concentrations during Endotoxin Shock in Dogs. *Eur. J. Pharmacol* 1991;205:277–282. [PubMed: 1667911]
- Odoux C, Crestani B, Lebrun G, Rolland C, Aubin P, Seta N, Kahn MF, Fiet J, Aubier M. Endothelin-1 Secretion by Alveolar Macrophages in Systemic Sclerosis. *Am. J. Respir. Crit. Care Med* 1997;156:1429–1435. [PubMed: 9372656]
- O'Sullivan ML, O'Grady MR, Minors SL. Plasma Big Endothelin-1, Atrial Natriuretic Peptide, Aldosterone, and Norepinephrine Concentrations in Normal Doberman Pinschers and Doberman Pinschers with Dilated Cardiomyopathy. *J. Vet. Intern. Med* 2007;21:92–99. [PubMed: 17338155]
- Peters IR, Peeters D, Helps CR, Day MJ. Development and Application of Multiple Internal Reference (housekeeper) Gene Assays for Accurate Normalization of Canine Gene Expression Studies. *Vet. Immunol. Immunopathol* 2007;117:55–66. [PubMed: 17346803]
- Prosek R, Sisson DD, Oyama MA, Biondo AW, Solter PF. Plasma Endothelin-1 Immunoreactivity in Normal Dogs and Dogs with Acquired Heart Disease. *J. Vet. Intern. Med* 2004;18:840–844. [PubMed: 15638267]
- Quan J, Uchida T, Takizawa S, Adur J, Nara E, Saida K. cDNA Cloning and Sequence Analysis of *Xenopus laevis* Preproendothelin-1. *J. Cardiovasc. Pharmacol* 2004;44 Suppl 1:S256–S259. [PubMed: 15838294]
- Rubens C, Ewert R, Halank M, Wensel R, Orzechowski HD, Schultheiss HP, Hoeffken G. Big Endothelin-1 and Endothelin-1 Plasma Levels are Correlated with the Severity of Primary Pulmonary Hypertension. *Chest* 2001;120:1562–1569. [PubMed: 11713135]
- Ruetten H, Thiemermann C. Endothelin-1 Stimulates the Biosynthesis of Tumour Necrosis Factor in Macrophages: ET-Receptors, Signal Transduction and Inhibition by Dexamethasone. *J. Physiol. Pharmacol* 1997;48:675–688. [PubMed: 9444616]
- Sakurai-Yamashita Y, Yamashita K, Yoshida A, Obana M, Takada K, Shibaguchi H, Shigematsu K, Niwa M, Taniyama K. Rat Peritoneal Macrophages Express Endothelin ET(B) but Not Endothelin ET(A) Receptors. *Eur. J. Pharmacol* 1997;338:199–203. [PubMed: 9456003]
- Sawamura T, Kimura S, Shinmi O, Sugita Y, Yanagisawa M, Masaki T. Analysis of Endothelin Related Peptides in Culture Supernatant of Porcine Aortic Endothelial Cells: Evidence for Biosynthetic Pathway of Endothelin-1. *Biochem. Biophys. Res. Commun* 1989;162:1287–1294. [PubMed: 2669747]
- Shimada K, Yonetani Y, Kita T, Suzumura A, Takayanagi T, Nakashima T. Cyclooxygenase 2 Expression by Endothelin-1-Stimulated Mouse Resident Peritoneal Macrophages in Vitro. *Eur. J. Pharmacol* 1998;356:73–80. [PubMed: 9761426]

- Speciale L, Roda K, Saresella M, Taramelli D, Ferrante P. Different Endothelins Stimulate Cytokine Production by Peritoneal Macrophages and Microglial Cell Line. *Immunology* 1998;93:109–114. [PubMed: 9536126]
- Teder P, Noble PW. A Cytokine Reborn? Endothelin-1 in Pulmonary Inflammation and Fibrosis. *Am J Respir Cell Mol. Biol* 2000;23:7–10. [PubMed: 10873147]
- Tessier-Vetzel D, Tissier R, Chetboul V, Carlos C, Nicolle A, Benbaron D, Dandrieux J, Thoulon F, Carayon A, Pouchelon JL. Diagnostic and Prognostic Value of Endothelin-1 Plasma Concentrations in Dogs with Heart and Respiratory Disorders. *Vet. Rec* 2006;158:783–788. [PubMed: 16766723]
- Uchide T, Saida K. Elevated Endothelin-1 Expression in Dogs with Heartworm Disease. *J. Vet. Med. Sci* 2005;67:1155–1161. [PubMed: 16327228]
- Wahl JR, Goetsch NJ, Young HJ, Van Maanen RJ, Johnson JD, Pea AS, Brittingham A. Murine Macrophages Produce Endothelin-1 After Microbial Stimulation. *Exp. Biol. Med. (Maywood)* 2005;230:652–658. [PubMed: 16179733]
- Wanecek M, Weitzberg E, Rudehill A, Oldner A. The Endothelin System in Septic and Endotoxin Shock. *Eur. J. Pharmacol* 2000;407:1–15. [PubMed: 11050285]
- Wang H, Quan J, Kotake-Nara E, Uchide T, Andoh T, Saida K. cDNA Cloning and Sequence Analysis of Preproendothelin-1 (PPET-1) from Salmon, *Oncorhynchus Keta*. *Exp. Biol. Med. (Maywood)* 2006;231:709–712. [PubMed: 16740985]
- Weitzberg E, Lundberg JM, Rudehill A. Elevated Plasma Levels of Endothelin in Patients with Sepsis Syndrome. *Circ. Shock* 1991;33:222–227. [PubMed: 2065442]
- Wellman ML, Krakowka S, Jacobs RM, Kociba GJ. A Macrophage-Monocyte Cell Line from a Dog with Malignant Histiocytosis. *In Vitro Cell. Dev. Biol* 1988;24:223–229. [PubMed: 3350786]
- Xu J, Zhong NS. The Interaction of Tumour Necrosis Factor Alpha and Endothelin-1 in Pathogenetic Models of Asthma. *Clin. Exp. Allergy* 1997;27:568–573. [PubMed: 9179432]
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A Novel Potent Vasoconstrictor Peptide Produced by Vascular Endothelial Cells. *Nature* 1988;332:411–415. [PubMed: 2451132]
- Yasuda N, Tsukui T, Masuda K, Kawai S, Ohmori K, Maeda S, Tsujimoto H. Cloning of the cDNA Encoding Canine Endothelin Receptors and their Expression in Normal Tissues. *J. Vet. Med. Sci* 2005;67:1075–1079. [PubMed: 16276069]
- Yildirim Y, Gunel N, Coskun U, Sancak B, Bukan N, Aslan S, Cetin A. Serum Big Endothelin-1 Levels in Female Patients with Breast Cancer. *Int. Immunopharmacol* 2008;8:1119–1123. [PubMed: 18550016]
- Zeihner AM, Goebel H, Schachinger V, Ihling C. Tissue Endothelin-1 Immunoreactivity in the Active Coronary Atherosclerotic Plaque. A Clue to the Mechanism of Increased Vasoreactivity of the Culprit Lesion in Unstable Angina. *Circulation* 1995;91:941–947. [PubMed: 7850978]
- Zhang J, Leontovich A, Sarras MP Jr. Molecular and Functional Evidence for Early Divergence of an Endothelin-Like System during Metazoan Evolution: Analysis of the Cnidarian, *Hydra*. *Development* 2001;128:1607–1615. [PubMed: 11290299]

Fig.1.

The constitutive and LPS-induced production of ET-1 by DH82 cells. (A) DH82 cells were incubated in medium alone or in the presence of 100 ng / ml LPS for increasing periods of time. (B) DH82 cells were incubated for 6 hrs in medium alone, or increasing concentrations of LPS. At the indicated times, culture supernatants were collected and assayed for ET-1 peptide concentration using a commercially available EIA kit. Peptide values represent the mean ($\pm$ SD) of triplicate determinations. Each figure is representative of three individual experiments, each performed in triplicate.

Fig. 2.

The effect of microbial challenge on DH82 ET-1 production. DH82 cells were incubated for 6 hours in either medium alone ("Control"), 100 ng / ml of LPS, or the indicated bacteria. Culture supernatants were collected and assayed for ET-1 peptide concentration using a commercially available EIA kit. Peptide values represent the mean ($\pm$ SD) of triplicate determinations. This figure is representative of three individual experiments, each performed in triplicate. The symbol " * " indicates significant changes ($P < 0.004$) in ET-1 production relative to the control.

Fig. 3.

The secretion of big ET-1 by DH82 cells. DH82 cells were cultured in medium alone or 1000 ng / ml of LPS, in the presence or absence of the ECE-1 inhibitor SM-19712. The concentrations of ET-1 (A) and big ET-1 (B) were determined using commercially available EIA kits. Peptide values represent the mean ($\pm$ SD) of a single experiment performed in triplicate. The symbols “* “ and “**” indicate significant changes ($P < 0.001$) in ET-1 or big ET-1 production relative to cells receiving no LPS or SM-19712 or cells receiving LPS, but no SM-19712, respectively.

Fig. 4.

Expression of mRNAs encoding the endothelin axis by DH82 cells. DH82 cells were cultured for 6 hours in medium alone or 1000 ng / ml of LPS. Monolayers were washed and total cellular RNA harvested. Reverse transcriptase PCR analysis was performed to amplify transcripts encoding preproendothelin-1 (ET-1), endothelin-converting enzyme-1 (ECE-1), endothelin receptor A (ETR-A), endothelin receptor B (ETR-B), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR primers used are described in Table I. Identical expression patterns were observed following RNA extraction from LPS treated and untreated cultures on three separate occasions.

Table 1

Canine primer sequences, along with their references (or NCBI Nucleotide accession numbers), and PCR product sizes in base pairs

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Reference / Accession number
GAPDH	TCAACGGATTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	90	Peters et al., 2007
ET-1	GGACAGCAGGGGGGAGAA	GTTGACCCAGATGATGTCAAGGT	130	Uchide and Saida, 2005
ECE-1	TGGGGGACTTTCAGCAACCT	GAGTGTCTGGAAGTTGTCTTTG	235	DQ195100
ETR-A	CCTTTGGTGTGCACTGCAATC	CGGTTCTTGTCATCTCATC	221	Yasuda et al., 2005
ETR-B	GGTGTCTGTCTAGTGTTTCG	GCAATCTGCATGCCACTCTTC	612	Yasuda et al., 2005