BINDING OF ENDOTHELIN-1 TO HUMAN BLOOD MONOCYTE
(Ikatan Endothelin-1 pada monosit darah manusia)

[1]Dr. med. Tri Hanggono Achmad, dr and [2]Prof. Dr. Govind S. Rao

[1]Bagian Biokimia, Fakultas Kedokteran – Universitas Padjadjaran, Bandung, Indonesia

[2]Institute of Clinical Biochemistry, University of Bonn, Bonn Germany

Korespondensi:

Dr. med. Tri Hanggono Achmad, dr

Bagian Biokimia, Fakultas Kedokteran Universitas Padjadjaran

Jl. Raya Bandung-Sumedang, Km 21, Jatinangor, Sumedang

Tilpon: 022-7794560, fax: 022-7795595

e-mail: tachmad@fk.unpad.ac.id

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[1]Tri Hanggono Achmad and [2]Govind S. Rao

[1]Department of Biochemistry, Faculty of Medicine – Universitas Padjadjaran, Bandung, Indonesia

[2]Institute of Clinical Biochemistry, University of Bonn, Bonn Germany

ABSTRACT

Endothelin-1 (ET-1) is a potent chemoattractant to human blood monocytes. To explore the presence of ET-1 receptor(s) on the monocyte, we studied the binding of ET-1 to freshly isolated human blood monocytes. Radioligand binding studies revealed the presence of two distinct subclasses of binding sites with apparent dissociation constants, K_ds, of 10.3 pM and 3.5 nM and maximal binding capacities, B_{max}s, of 0.027 fmol and 0.63 fmol/1.5x10^5 cells. Using monocyte migration as a response to ET-1, and ET-1 receptor antagonists BQ-123, BQ-18257B and IRL-1038, the presence of two ET receptor subtypes, ET_A and ET_B, were detected. These results suggest that the chemotactic stimulus introduced by ET-1 maybe activating ET-1 specific receptors on the monocytes.

Key words: Endothelin-1, monocyte, receptor-binding

IKATAN ENDOTHELIN-1 PADA MONOSIT DARAH MANUSIA

[1]Tri Hanggono Achmad dan [2]Govind S. Rao

[1]Bagian Biokimia, Fakultas Kedokteran – Universitas Padjadjaran, Bandung, Indonesia

[2]Institute of Clinical Biochemistry, University of Bonn, Bonn Germany

ABSTRAK

Endothelin-1 (ET-1) dikenal sebagai *chemoattractant* poten bagi monosit. Untuk mengungkapkan adanya reseptor ET-1 pada monosit, dilakukan penelitian ikatan ET-1 pada monosit yang diisolasi dari darah manusia. Penilaian ikatan *radioligand* menunjukkan adanya dua sub-kelas berbeda dari tempat ikatan dengan konstanta disosiasi (K_d) masing-masing 10,3 pM dan 3,5 nM, dan kapasitas ikatan maksimal (B_{max}) masing-masing sebesar 0,027 fmol dan 0,63 fmol/1.5x10^5 sel. Dari hasil penilaian tingkat migrasi monosit sebagai respon terhadap ET-1 dengan atau tanpa beberapa antagonis reseptor ET-1, BQ-123, BQ-18257B dan IRL-1038, terdeteksi adanya dua subtype reseptor ET, yaitu ET_A dan ET_B. Hasil ini menunjukkan bahwa rangsangan kemotaksis yang ditimbulkan ET-1 dapat mengaktifkan reseptor spesifik ET-1 pada monosit.

Kata kunci: Endothelin-1, monosit, ikatan-reseptor

INTRODUCTION

Endothelin (ET-1) is a potent vasoconstrictor peptide consisting of 21 amino acids; it was first isolated from cultured porcine endothelial cells[1]. Since this initial discovery, cells derived from many different tissues have been found to synthesize and secrete the peptide[2-3]. Subsequently, the cloning and sequencing of endothelin (ET) related genes revealed the existence of two additional peptides, ET-2 and ET-3[4]. ETs have variety of biological actions and have been implicated in the pathogenesis of many diseases, such as atherosclerosis[5,6]. In the latter disease, monocyte attachment to the endothelium and migration into the vessel intima are the initiating steps in atherogenesis[7]. We found that ET-1 is a strong chemoattractant to blood monocytes [8], supportive of the role of ET-1 in the pathogenesis of atherosclerosis. ETs initiate cellular effects and physiological actions after binding to cell surface membrane receptors. Changes in intracellular Ca^{2+}, c-AMP, c-GMP and activation of protein kinases are some of the responses subsequent to ligand-receptors is not known. The present study was designed to explore the occurrence of binding sites for ET-1 on human blood monocytes and to study the effect of receptor antagonists on monocyte migration. The results show that human monocytes possess at least two distinct subtypes of specific receptors for ET-1, and ET-1 receptor antagonists lead to decreases in monocyte migration.

MATERIALS AND METHODS

The materials used were obtained from the following sources: Twenty four-well plates (Cat.No. 3047) and cell culture insert (Cat.No.3095) from Becton Dickinson, Heidelberg; NycoPrep 1068 and NycoPrep 1063 from Nycomed Pharma, Oslo, Norway; human serum albumin from Behringwerke AG, Marburg; Dextran T-500 from Biozym Diagnostik, Hameln. Mayer's hemalum solution from Merck, Darmstadt; N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic

acid (HEPES) from Serva, Heidelberg; fetal calf serum and Medium RPMI 1640 from Biochrom, Berlin; Endothelin-1 human (synthetic) and penicillin-streptomycin from Sigma, ^{125}I-ET-1 (specific radioactivity 2000 Ci/mmol) from Amersham and endothelin receptor antagonist BQ-123, BE-18257B and IRL-1038 from Alexis Corporation, Switzerland.

Monocyte Isolation

Human monocytes were isolated from freshly drawn blood in EDTA (1.6 mg/ml) Monovettes (Sarstedt, Numrecht); details have been described previously [8].

Binding Assay

To determine the binding of ^{125}I-ET-1 to monocytes as a function of time, freshly isolated monocytes (150,000 cells / 100µl) were incubated for various lengths of time with ^{125}I-ET-1 (3 x 10^{10}M) in the absence or in the presence of 1µM of unlabeled ET-1; the total volume was 200µl and the temperature was 37^0C. The microcentrifuge tube (capacity 400 µl, Beckman, Munich) containing the incubation contents was on a layer of a mixture of 100 µl of silicone oil (Silicon oil AR 20:AR 200/1:1,v/v). The incubation was terminated by centrifugation of the tubes at 10,000g for 10s. The monocytes formed a pellet under the silicone oil layer. The tubes were cut at the oil level and the pellet was taken for measurement of radioactivity in a gamma counter (Packard, Cobra Auto-Gamma); the counting efficiency was 74%. Dependence of binding on the concentration of ^{125}I-ET-1, in the absence and in the presence of 1 µM of unlabeled ET-1, was determined as described above. Displacement of the radioligand from its binding sites was evaluated by incubating the monocytes with a constant concentration of ^{125}I-ET-1 (5 x 10^{10}M) and increasing concentrations of unlabeled ET-1 and processed as mentioned above. Specific binding was defined as total binding minus non-specific binding that occurred in the presence of

an excess (1µM) of unlabeled ET-1. The dissociation constant, K*d*, and the maximal binding capacity, B*max*, were determined using the Ligand program developed by Munson and Rodbard[9].

Chemotaxis assay

Chemotaxis of monocytes was assayed by the method described previously[8] in a24-well plate with cell culture inserts. The bottom wells (500 µl) contained various concentrations of ET-1 dissolved in RPMI 1640 medium. Monocytes were added to the upper wells (cell culture insert) in a volume of 250 µl which contained 1.25 x 10^5 cells per well. After 60 min of incubation at 37^0C in a humidified incubator with air and 5% CO_2, the upper wells were removed and the non-migrated cells, i.e., the cells that did not settle on the membrane-bottom of the well, were removed gently by sucking up with a pipette. Cells on the membrane were carefully washed with PBS to remove the rest of the cells which were not firmly attached. They were then fixed with methanol and stained with Mayer's hemalum solution for 7 min. The cells firmly attached to the membrane were counted with a 12.5-fold ocular and a 10-fold objective with four counting grids. Five areas containing 4 grids were counted per well and averaged. The mean variation among the counted field was 12 ± 6% for the test solutions and 19 ± 8% for the controls. Chemotaxis activity is expressed as chemotactic index, CI, defined as the ratio of the number of cells migrating in the response to ET-1 to the number of cells migrating when only medium was in the lower chamber, which was also the control. Receptor subtype was evaluated by incubating the monocytes with specific endothelin receptor antagonists BQ-123 (0.1 and 10 µM), BE-18257B (1 µM) or IRL-1038 (1 µM) for 60 min followed by determination of chemotaxis toward ET-1 as described above.

Results

The binding of ^{125}I-ET-1 to monocytes was time dependent; it increased progressively up to 60 min and reached an apparent steady state thereafter (Fig.1); non-specific binding varied between 30% and 50% of total binding over the time range.

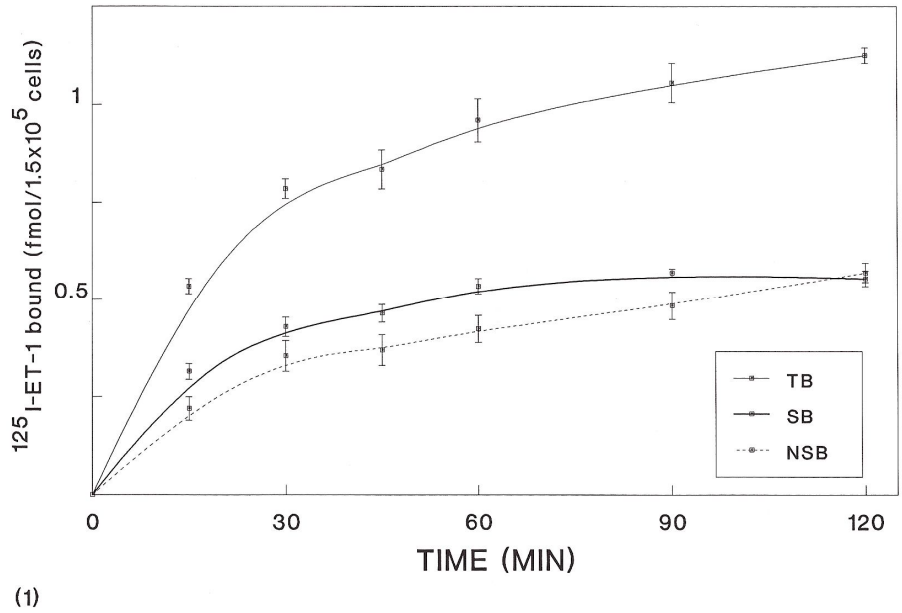


FIGURE 1. Specific binding of ^{125}I-ET-1 to human blood monoctye as a function of time.
Freshly isolated monoctyes were incubated with 3 x 10^{-10} M ^{125}I-ET-1 at 37^{0}C for the times indicated. Specific binding (SB) was obtained by substracting non-specific binding (NSB) that occurred in the presence of 1 µM unlabeled ET-1 from total binding (TB). Each point is the mean ± SD of triplicate experiments.

Binding of ^{125}I-ET-1 increased with increasing concentrations of ^{125}I-ET-1, the kinetics of which appeared to deviate from the hyperbolic form of a typical concentration curve (Fig.2). Analysis of the binding data by the Ligand program (Fig.2 inset) revealed the presence of two distinct subclasses of binding sites. One had a high affinity and low binding capacity for ET-1, the apparent K*d* was 10.3 pM and the B*max* 0.027 fmol/1.5 x 10^5 cells. The second subclass of binding site had low affinity and high binding capacity for ET-1; the apparent K*d* and B*max* values were 3.5 nM and 0.63 fmol/1.5 x 10^5 cells, respectively.

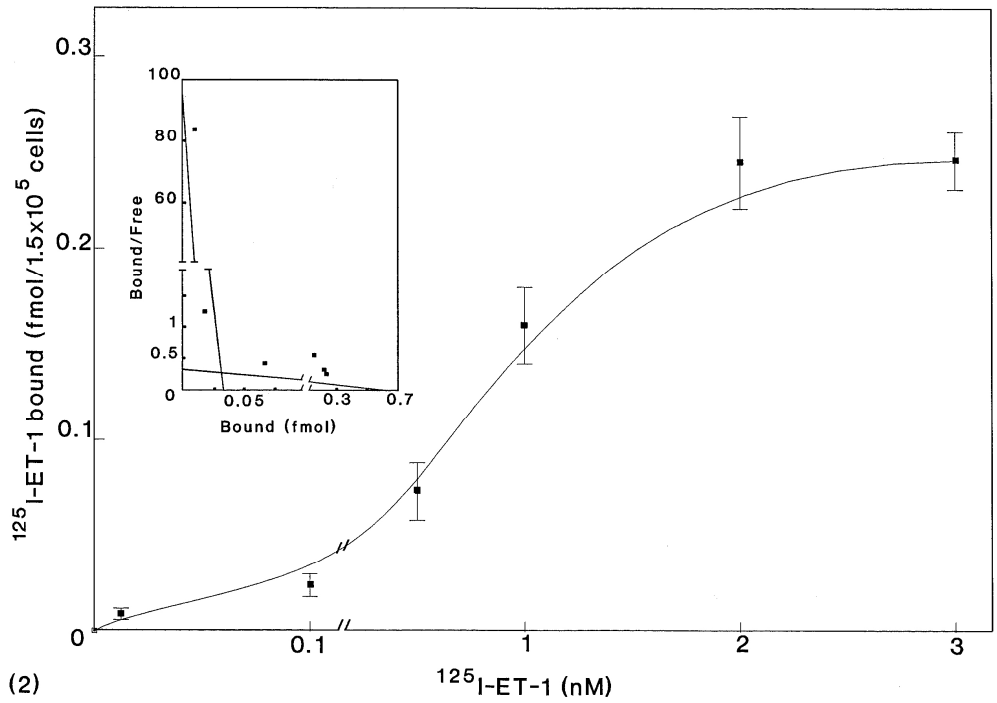


FIGURE 2. Saturable binding of ^{125}I-ET-1 to human blood monocyte. Freshly isolated monocytes were incubated at 37^{0}C for 60 min with various concentration of ^{125}I-ET-1. Non-specific binding in the presence of 1 µM unlabeled ET-1 ranged from 30%-50% of total binding. Each point is the mean ± SD of triplicate experiments. Inset shows the Scatchard plot of the binding data.

Unlabeled ET-1 competitively displaced ^{125}I-ET-1 from its binding sites on the monocyte; the calculated inhibitor constants, K*i*s, [(EC_{50}/(1 + L/K)] for the two binding sites, calculated by the Ligand program, were 70 pM and 3.6 nM, respectively (Fig.3).

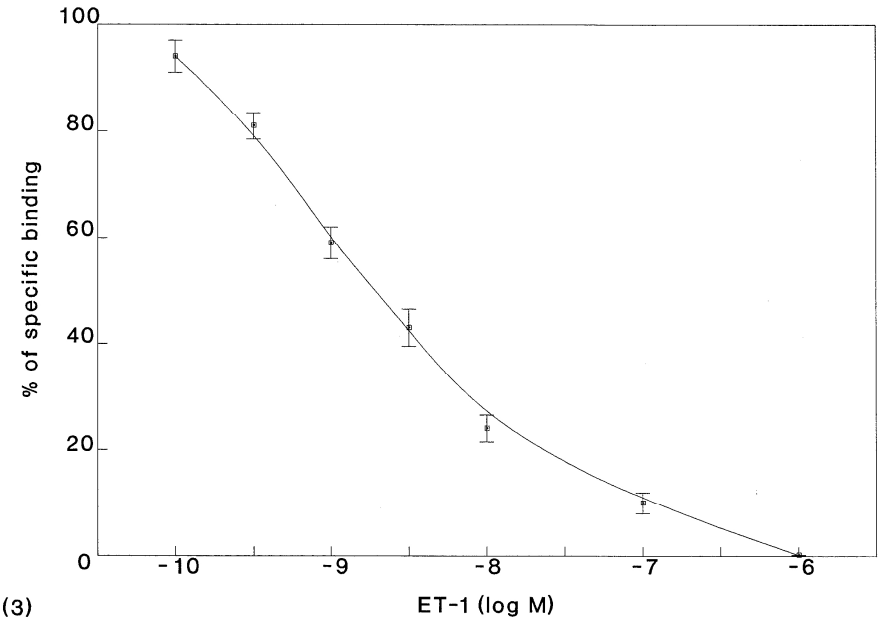
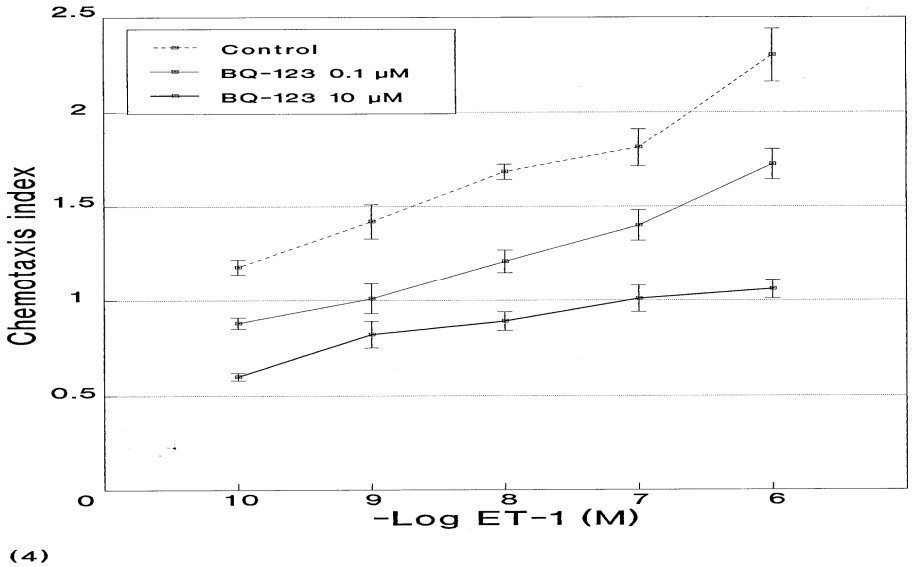


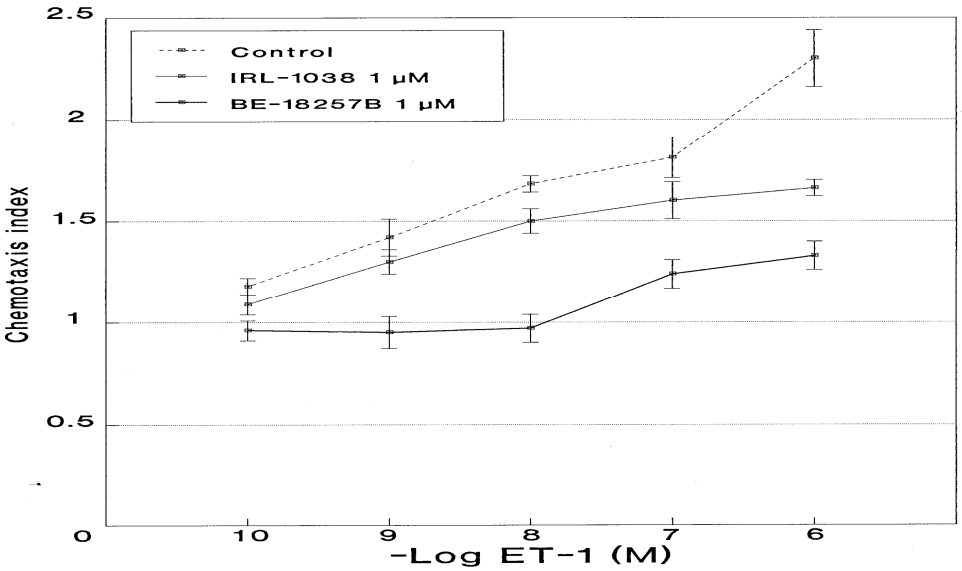
FIGURE 3. Displacement of ^{125}I-ET-1 from its binding sites on monocytes by unlabeled ET-1. 1.5 x 10^5 freshly isolated monoctyes were incubated with ^{125}I-ET-1 (5 x 10^{-10}M) in a total volume of 200 µl in the absence and presence of increasing concentrations of unlabeled ET-1. Each point is the mean ± SD of triplicate experiment.

Chemotaxis of monocytes toward varying concentrations, 10^{-10} – 10^{-6}M, of ET-1 and in the presence of 2 fixed concentrations of the specific ET_A receptor antagonist, BQ-123, decreased migration (Fig.4). At 0.1 µM of BQ-123 the decrease in CI ranged from 22% to 29%, at 10 µM the decrease ranged from 42% to 54%. Another ET_A receptor antagonist, BE-18257B (1 µM), also inhibited monocyte migration; the decrease in CI ranged from 20% to 35%. The ET_B receptor antagonist IRL-1038 (1 µM) reduced migration; the decrease in the CI ranged from 8% to 28% (Fig.5).



(4)

FIGURE 4. Influence of ET_A receptor antagonist BQ-123 on the chemotaxis of monocytes. The monocytes were pretreated with two different concentrations of BQ-123 for 60 min prior to the chemotaxis assay. The concentration range of ET-1 was 10^{-10}M-10^{6}M. The values are means and the vertical bars SDs from 3 separate experiments.



(5)

FIGURE 5. Influence of ET_A receptor antagonist BQ-1825B, and ET_B receptor antagonist IRL-1038 on the chemotaxis of monocytes. Treatment of monocytes with the receptor antagonists and the chemotaxis assay were performed as described in the legend for Fig.3. The values are means and the vertical bars the SDs from 3 separate experiments.

DISCUSSION

Monocyte attachment to the endothelium and migration into the intimal space of the blood vessel are construed as the earliest events in the chain of reactions leading to atherosclerotic changes of the vasculature. In patients with risk factors for atherosclerosis the circulating levels of ET-1 are increased[10,11]. Our previous study indicated that ET-1 possesses chemotactic activity for human blood monocytes. The underlying mechanism triggering migration was through Ca^{2+} influx since Ca^{2+} antagonists reduced migration[8]. In the present study we used ^{125}I-ET-1 to detect binding sites for ET-1 on the monocyte. The results clearly demonstrate, for the first time, the occurrence of two distinct subpopulations of binding sites for ET-1 on freshly isolated human bbod monocytes. Recent studies have reported that at least two subtypes of binding sites for ET-1 isopeptides exist on the membrane endothelial cells[12]. The binding of ET-1 to blood monocytes was reversible and the affinity of the binding site with the low K*d* lies in the pM range agreeing with the concentration of ET-1 in plasma of normal individuals (0.1 -7.98 pM)[13]. The high affinity component possessed 108 sites and the low affinity component 2529 sites per monocyte. The binding affinity of ET-1 to cells from different tissues [14,15] and the binding affinity of ET-1 to monocytes is almost similar, ranging from pM to nM. To date three ET receptors, ET_A, ET_B and ET_C, have been identified and several ET receptors antagonists have studied[16-18]. Using the chemotaxis as a response of monocytes to ET-1 and specific ET-1 receptor antagonists our study shows that at least two receptor subtypes are activated on the monocyte during ET-1 stimulus; one is the ET_A receptor which is affected by the antagonist BQ-123 and BE-18257 and the second one is the ET_B receptor which is affected by the antagonist IRL-1038. The consequence of receptor antagonism was decrease in the number of monocytes migrating in response to the chemotactic stimulus of ET-1. Apparently both receptors contribute to the chemotaxis process. Considering the affinities of the two receptor subtypes and the decrease in monocyte migration

affected by the receptor antagonists, it is likely that the high affinity component is the ET_A receptor and the binding component with the low affinity the ET_B receptor, since ET-1 was bound with a higher affinity to the ET_A than to the ET_B receptor. The data also suggest that the ET_A receptors exercises a greater influence on chemotaxis than the ET_B recetors albeit their greater abundance. The different affinities and capacities of the endothelin receptors suggest that endothelin isopeptides are involved in a wide range of physiological and pathological events in an organism or cells, as well as in monocytes[19]. In conclusion, our data provide evidence for the occurrence of specific receptors for ET-1 on human blood monocytes. Redistribution or a change in directional orientation of these receptors by ET-1 probably enhances monocyte migration.

REFERENCES

1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, *et al.* A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988; 332: 411-415.
2. Sanz MJ, Johnston B, Issekutz A, Kubes P. 1999. Endothelin-1 causes P-selectin-dependent leukocyte rolling and adhesion within rat mesenteric microvessels. Am. J. Physiol. 1999; 277: H1823-H1830.
3. Morawietz H, Duerrschmidt N, Niemann B, Galle J, Sawamura T, Holtz J. Induction of the oxLDL receptor LOX-1 by endothelin-1 in human endothelial cells. Biochem. Biophys. Res. Commun. 2001; 284(4): 961-965.
4. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, *et al.* The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. USA., 1989; 86: 2863-2867.

5. Kinlay S, Behrendt D, Wainstein M, Beltrame J, Fang JC, Creager MA, *et al.* Role of endothelin-1 in the active constriction of human atherosclerotic coronary arteries. Circulation. 2001; 104(10): 1114-1118.
6. Bousette N, Gaid A. Endothelin-1 in atherosclerosis and other vasculopathies. Canadian Journal of Physiology and Pharmacology. 2003; 81(6): 578-87.
7. Ross R. Atherosclerosis is an inflammatory disease. Am. Heart J. 1999; 138: S419-S420.
8. Achmad TH, Rao GS. Chemotoxis of human blood monocytes toward endothelin-1 and the influence of calcium channel blockers. Biochem. Biophys. Res. Commun. 1992;189: 994-1000.
9. Munson PJ. Rodbard D. A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 1980; 107: 220-239.
10. Kahler J, Ewert A, Weckmuller J, Stobbe S, Mittmann C, Koster R, et al. Oxidative stress increases endothelin-1 synthesis in human coronary artery smooth muscle cells. J. Cardiovasc. Pharmacol. 2001; 38(1): 49-57.
11. Ihling C, Szombathy T, Bohrmann B, Brockhaus M, Schaefer HE, Loeffler BM. Coexpression of endothelin-converting enzyme-1 and endothelin-1 in different stages of human atherosclerosis. Circulation. 2001; 104(8): 864-869
12. Kobayshi T, Miyauchi T, Sakai S, Maeda S, Yamaguchi I, Goto K, et al. Down-regulation of ET^sub B^ receptor, but not ET^sub A^ receptor, in congestive lung secondary to heart failure. Are marked increases in circulating endothelin-1 partly attributable to decreases in lung ET^sub B^ receptor-mediated clearance of endothelin-1? Life Sci. 1998; 62(2): 185-193.
13. Shichiri M, Hirata Y, Ando K, Emori T, Ohta K, Kimoto S, et al. Plasma endothelin levels in hypertension and chronic renal failure. Hypertension. 1990; 15: 493-496.

14. Housset C, Rockey DC, Bissel DM. Endothelin receptors in rat liver. Proc. Natl. Acad. Sci. USA. 1993; 90: 9266-9270.
15. Perreault T, Berkenbosch JW, Barrington KJ, Decker ER, Wu C, Brock TA. TBC3711, an ET^sub A^ receptor antagonist, reduces neonatal hypoxiainduced pulmonary hypertension in piglets. Pediatr. Res. 2001; 50(3): 374-383.
16. DiCarlo VS, Chen SJ, Meng QC, Durand J, Yano M, Chen YF, *et al*. 1995, ET^sub A^-receptor antagonist prevents and reverses chronic hypoxiainduced pulmonary hypertension in rat. Am. J. Physiol. 1995; 269: L690-L697.
17. Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B, *et al.* 2001. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. Clin. Pharmacol. Ther. 2001; 69(4): 223-231.
18. Luscher TF, Barton M. Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. Circulation. 2000; 102(19): 2434-2440.
19. Haug C, Schmid-Kotsas A, Zorn U, Schuett S, Gross HJ, Gruenert A, *et al.* 2001. Endothelin-1 synthesis and endothelin B receptor expression in human coronary artery smooth muscle cells and monocyte-derived macrophages is upregulated by low density lipoproteins. J. Mol. Cell Cardiol. 2001; 33(9): 1701-1712.