

Original article

Cardiotrophin-1 induces intercellular adhesion molecule-1 expression by nuclear factor κ B activation in human umbilical vein endothelial cells

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Keywords: *cardiotrophin-1; intercellular adhesion molecule-1; nuclear factor κ B; human umbilical vein endothelial cells*

Background In addition to elevated concentrations of cytokines, patients with congestive heart failure (CHF) show endothelial dysfunction and increased plasma concentrations of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1). Furthermore, the concentration of cardiotrophin-1 (CT-1) – a cytokine of the interleukin-6 superfamily – is increased in CHF. We tested the hypothesis whether CT-1 is able to induce ICAM-1 in human umbilical vein endothelial cells (HUVEC). Furthermore we examined the signalling mechanisms of CT-1 mediated ICAM-1 expression.

Methods Confluent layers of HUVEC were incubated with increasing concentrations of CT-1 (5 to 100 ng/ml) for different periods. ICAM-1 mRNA was determined by real-time polymerase chain reaction (PCR) and ICAM-1 surface expression by fluorescence-activated cell sorter (FACS) analysis and soluble ICAM-1 (sICAM-1) in the culture supernatant by enzyme linked immunosorbent assay (ELISA). To clarify the signalling pathway of CT-1 induced ICAM-1 expression we used various inhibitors of possible signal transducing molecules, electromobility shift assay (EMSA) and Western blot analysis.

Results CT-1 induced ICAM-1 mRNA (1.8 ± 0.8 fold increase compared to unstimulated cells after 6 hours) and protein (1.4 ± 0.2 fold increase compared to unstimulated cells after 48 hours) in HUVEC in a time- and concentration-dependent manner. EMSA experiments show that CT-1 causes nuclear factor (NF) κ B activation. Because parthenolide could inhibit CT-1 induced ICAM-1 expression NF κ B activation is required in this pathway. CT-1 did not activate extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38.

Conclusion CT-1 is able to induce ICAM-1 in endothelial cells by NF κ B activation. These results may explain in part elevated ICAM-1 concentrations in patients with CHF and endothelial dysfunction.

Chin Med J 2008;121(24):2592-2598

Cardiotrophin-1 (CT-1) is a 201 amino acid member of the interleukin-6 (IL-6) superfamily. CT-1 was discovered by Pennica et al¹ in 1995 via expression cloning of mouse embryoid bodies. CT-1 is highly expressed in the myocardium during cardiogenesis.¹⁻³ Furthermore in fetal life CT-1 is found in the kidney and lung and later in skeletal muscle, ovary, testis, prostate and colon.¹ CT-1 induces myocyte hypertrophy leading to an increase in myocyte size by adding sarcomeres in series rather than in parallel.^{1,2} The biological effects of CT-1 are multiple. CT-1 not only causes myocardial hypertrophy but also promotes myocyte survival during embryogenesis⁴ and is involved in scar formation and angiogenesis in the case of myocardial infarction.⁵ There are several studies which could show that CT-1 is increased in patients with CHF and produced in the failing ventricle.⁶⁻⁸

Furthermore, CT-1 is involved in neuroprotection,⁹ causes growth of the liver, spleen and kidney when administered intraperitoneally in a mouse model and increases the number of platelets and red blood cells.¹⁰

CT-1 binds to the leukemia inhibitory factor (LIF) receptor which causes a heterodimerisation with

glycoprotein (gp) 130. The intracellular signalling pathway consists of the extracellular signal regulated kinases (ERK), the mitogen activated protein (MAP) kinases, the janus kinase (JAK)/signal transducers and activators of transcription (STAT) system, and phosphoinositid (PI) 3-kinase¹¹⁻¹³ leading to the activation of multiple transcription factors like STAT3, NF κ B and heat shock proteins.

In the last decades we have learned that in congestive heart failure (CHF) not only reduced exercise capacity and left ventricular function is found but also a state of chronic inflammation with vascular dysfunction and endothelial activation.¹⁴⁻¹⁸ There is growing evidence that an abnormal inflammatory response including overexpression of proinflammatory cytokines, soluble adhesion molecules and chemoattractant factors may be

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involved in the progression of CHF.^{19,20} In CHF some proinflammatory cytokines are produced in the myocardium and can spill over into circulation where they can secondarily activate cells.^{21,22} This may explain the fact that several investigators could show an abundance of endothelial adhesion molecules in dilatative cardiomyopathy.²³⁻²⁶ Under these adhesion molecules intercellular adhesion molecule-1 (ICAM-1) is upregulated in CHF.²⁷ ICAM-1 is a member of the immunoglobulin gene superfamily which is found on the surface of macrophages and endothelial cells and mediates the adhesion of inflammatory cells to the endothelium.²⁸ Furthermore it could be shown that increased ICAM-1 concentrations are found in CHF and are related to clinical outcome.²⁹

In this study we investigated whether CT-1 is able to induce ICAM-1 expression in HUVEC and examined the pathway responsible.

METHODS

Reagents

Recombinant human CT-1 was purchased from R&D Systems (Wiesbaden, Germany) and dissolved according to the manufacturer's instructions. Parthenolide was purchased from Sigma chemicals (Deisenhofen, Germany), SP600125 from Stressgen (Victoria, Canada), U0126 from Biomol (Plymouth Meeting, USA) and SB203580 from Upstate (Dundee, UK). The blocking antibody against CT-1 was purchased from R&D Systems.

To determine MAP kinase activation by Western blot analysis we used a commercially available set of antibodies: pERK, ERK, pJNK, JNK, pp38 and p38 (Becton Dickinson/Pharmingen, Heidelberg, Germany).

Cell culture

Primary cultures from human vein endothelial cells were purchased from PromoCell (Heidelberg). Cell culture was done according to the manufacturer's manual in endothelial growth medium with 2% fetal calf serum (EGM, PromoCell, Heidelberg). Cells were grown to confluence in collagen I coated tissue culture plastic (Becton Dickinson, Franklin Lakes, USA). Cells were used in the second to fifth cell passages. Pharmacological agents, dissolved in fresh medium, were added to confluent cell monolayers for defined time intervals and concentrations. As a control, fresh medium was added to the cells. All experiments were done with endothelium growth medium.

All stimulants, inhibitors and media had no significant level of endotoxin according to the manufacturers' instructions.

Real-time polymerase chain reaction (PCR)

Total RNA from cultivated HUVEC was extracted according to the RNeasy protocol (Qiagen, Hilden, Germany). One μg of total RNA was reversely transcribed into cDNA in a volume of 20 μl with avian

myeloma leukaemia virus (AMV) reverse transcriptase and oligo dT primers (Promega, Madison, USA) according to the manufacturer's manual.

Real-time PCR measurement of ICAM-1 cDNA was performed with the Light Cycler Instrument using the Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). For verification of the correct amplification product, PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. The specific primer pair for ICAM-1 was: sense primer 5'-TTGGAAGCCTCATCCG-3', antisense primer 5'-CAATGTTGCGAGACCC-3'. The amplification program for ICAM-1 consisted of 1 cycle of 95°C with a 30-second hold followed by 40 cycles of 95°C with a 5-second hold, 57°C annealing temperature with a 10-second hold and 72°C with a 20-second hold. The specific primer pair for GAPDH was: sense primer 5'-GGGAAGGTGAAGGTCCG-3', antisense primer 5'-TGGACTCCACGACGTACTCAG-3'. The amplification program for GAPDH consisted of 1 cycle of 95°C with a 30-second hold followed by 30 cycles of 95°C with a 5-second hold, 59°C annealing temperature with a 10-second hold and 72°C with a 20-second hold. Each reaction (20 μl) contained 2 μl cDNA, 2.5 mmol/L MgCl₂, 1 pmol of each primer and 2 μl of Fast Starter Mix (containing buffer, dNTPs, Sybr Green dye and Taq polymerase). Amplification was followed by melting curve analysis to verify the correctness of the amplicon. A negative control without cDNA was run with every PCR to assess the specificity of the reaction. Analysis of data was performed using Light Cycler software version 3.5. PCR efficiency was determined by analysing a dilution series of cDNA (external standard curve). The identity of the PCR product was confirmed by comparing its melting temperature (T_m) with the T_m of amplicons from standards or positive controls. GAPDH was analyzed in parallel to each PCR and the resulting GAPDH values were used as standards for presentation of ICAM-1 transcripts.

Fluorescence-activated cell sorter (FACS) analysis

HUVEC monolayers were incubated with CT-1 (50 ng/ml) for the indicated times. HUVEC without CT-1 application served as control. Afterwards cells were separated with Accutase (PAA Laboratories, Pasching, Austria). Then HUVECs were incubated for 30 minutes at 4°C with the specific PE-labelled monoclonal antibody against human ICAM-1 (Caltag, Hamburg, Germany) or control antibody of the appropriate isotype class but of irrelevant antigen specificity. Flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg) was performed with laser excitation at 488 nm. Mean fluorescence intensity was determined from at least 10 000 counted cells. Specific binding was determined by subtraction of the isotype control antibody.

Electrophoretic mobility shift assay (EMSA) for NF κ B

Nuclear extracts were achieved by the EpiQuikTM Nuclear Extraction KIT I (Epigentek, NY, USA)

according to the manufacturer's manual. Afterwards protein concentration of the nuclear extracts was determined. For determination of NF κ B 2 μ g of nuclear proteins were used and further analyzed by gel EMSA according to the supplier's manual. EMSA kits and probes were purchased from Panomics, Redwood City, USA. The intensity of the signal was determined by a LAS Science Imaging System (Fuji Film Life Science, Duesseldorf, Germany) and the software Phoretix 1D Advanced v4.00 (Biostep, Jahnsdorf, Germany).

Soluble ICAM-1 (sICAM-1)

After the indicated time sICAM-1 was determined with a commercially available quantitative sandwich enzyme immunoassay technique in the supernatant of confluent HUVEC stimulated with CT-1 according to the supplier's manual (Human soluble ICAM-1/CD54 Immunoassay, R&D Systems).

Statistical analysis

Because the amount of the cytokines produced was different in each experiment, the effects on ICAM-1 production were normalized to unstimulated cells, which were set as one. Data were analysed by non-parametric methods to avoid assumptions about the distribution of the measured variables. Comparisons between groups were made with the Wilcoxon test. Statistical analysis was done with WinSTAT for Excel. All values are reported as mean \pm standard deviation (SD). Statistical significance was considered to be indicated by a value of $P < 0.05$.

RESULTS

Concentration and time dependent induction of ICAM-1 mRNA in HUVEC by CT-1

In the first experiment we examined whether CT-1 is able to induce ICAM-1 in HUVEC. Confluent layers of HUVEC were stimulated with various concentrations of CT-1 for 6 hours. We found that CT-1 caused a concentration dependent induction of ICAM-1 mRNA measured by real-time-PCR. Maximal ICAM-1 expression was seen with 100 ng/ml CT-1 (Figure 1A). Our results show that there is a significant direct relation of CT-1 concentration in the medium with ICAM-1 mRNA in endothelial cells.

In the next sets of experiments we stimulated confluent layers of HUVEC with 50 ng/ml CT-1 for various periods. We found a significant increase of ICAM-1 mRNA after 6 hours. ICAM-1 mRNA was further increased up to 24 hours with no significant difference from 6 hours (Figure 1B). These data indicate that CT-1 is able to induce time-dependent ICAM-1 mRNA in HUVEC.

To exclude a nonspecific effect of CT-1 on ICAM-1 mRNA expression in HUVEC we used a blocking antibody against CT-1. The CT-1 antibody was able to block CT-1 induced ICAM-1 mRNA expression, whereas the antibody alone had no effect on ICAM-1 mRNA (data not shown).

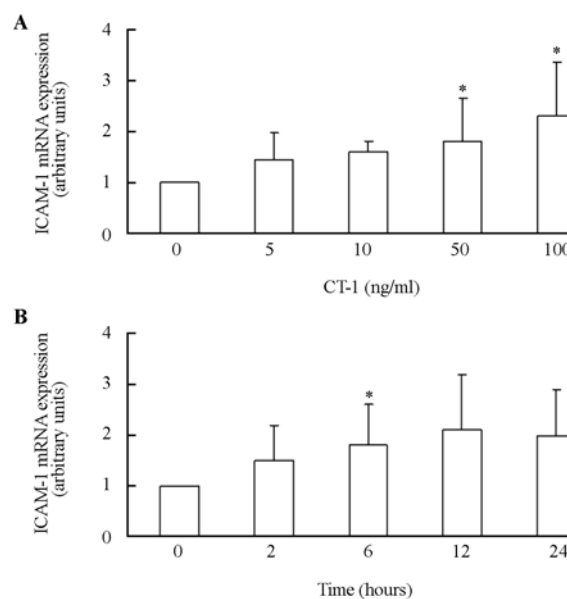


Figure 1. (A) Concentration dependent production of ICAM-1 mRNA by HUVEC after incubation with CT-1. Confluent monolayers of HUVEC were incubated with various concentrations of CT-1. After 6 hours RNA was isolated and ICAM-1 mRNA was determined by real-time-PCR. $n=6$, data are expressed as mean \pm SD. * $P < 0.05$ compared to unstimulated cells. (B) Time dependent expression of ICAM-1 mRNA in HUVEC after incubation with CT-1 (50 ng/ml). After the indicated time ICAM-1 protein was determined by real-time-PCR. $n=5$, data are expressed as mean \pm SD. * $P < 0.05$ compared to unstimulated cells.

Effect of CT-1 on ICAM-1 protein expression in HUVEC

FACS analysis was used to determine ICAM-1 surface expression after CT-1 application in HUVEC for various periods. CT-1 induced ICAM-1 in a time dependent manner (Figure 2A). Maximal surface expression of the ICAM-1 protein was achieved after 48 hours ((1.39 \pm 0.23)-fold compared to unstimulated cells). If ICAM-1 is also shed under culture conditions elevated sICAM-1 concentrations should be detectable after CT-1 application in the supernatant. Indeed after 24 and 48 hours CT-1 (50 ng/ml) incubation we found an increase of sICAM-1 in the supernatant after CT-1 stimulation compared to unstimulated HUVEC (Figure 2B). The fact that in the supernatant only a small increase of sICAM-1 is found represents that only a small amount of membrane bound ICAM-1 is cleaved and detectable in the medium. In summary CT-1 caused an increase of ICAM-1 protein in the cell membrane of HUVEC and this increase was accompanied by elevated concentration of sICAM-1 in the supernatant.

Signaling pathway of CT-1 induced ICAM-1 expression in HUVEC

The major intracellular signal transduction pathways involved in regulation of ICAM-1 expression include the mitogen activated protein kinases (ERK, JNK and p38) and the NF κ B signaling pathway.³⁰ Therefore we used different inhibitors: parthenolide for NF κ B inhibition,

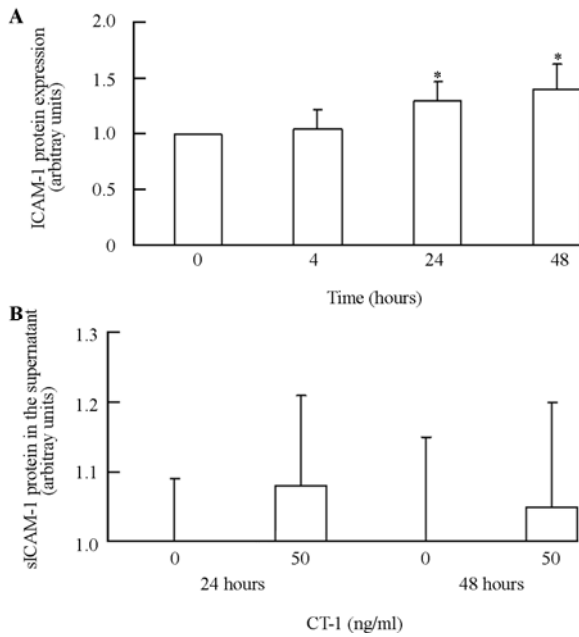


Figure 2. (A) Time dependent surface expression of ICAM-1 protein of HUVEC after incubation with CT-1 (50 ng/ml). After the indicated time ICAM-1 protein was determined by FACS analysis. *n*=5, data are expressed as mean ± SD. **P* < 0.05 compared to unstimulated cells. (B) Time dependent concentration of sICAM-1 protein in the supernatant of HUVEC after incubation with CT-1 (50 ng/ml). After the indicated time sICAM-1 protein was determined by ELISA.

U0126 for ERK inhibition, SP600125 for JNK inhibition and SB203580 for p38 inhibition. Confluent layers of HUVEC were incubated with CT-1 (50 ng/ml) for 6 hours in the presence of these inhibitors. After this period cells were lysed and ICAM-1 mRNA expression was determined by real-time-PCR. All inhibitors were applied 30 minutes before CT-1 application. The inhibitors alone did not influence basal ICAM-1 mRNA expression in HUVEC significantly (data not shown). Only parthenolide was able to inhibit CT-1 induced ICAM-1 mRNA in HUVEC indicating that NFκB is significantly responsible for ICAM-1 induction. Inhibition of ERK, JNK and p38 had no significant effect on CT-1 induced ICAM-1 mRNA induction (Figure 3). Piceatannol and AG490 both are STAT3 inhibitors, had no effect on CT-1 induced ICAM-1 expression indicating that STAT3 is not involved in the signalling way (data not shown).

To confirm our data we used EMSA. After 25 minutes stimulation with various concentrations of CT-1 nuclear extracts of HUVEC, a concentration dependent increase of NFκB nuclear translocation was shown (Figure 4), indicating that CT-1 causes NFκB nuclear translocation. This translocation could be inhibited by parthenolide indicating that CT-1 induced ICAM-1 expression is NFκB dependent (Figure 5).

DISCUSSION

In the last decades the pathophysiological concepts of

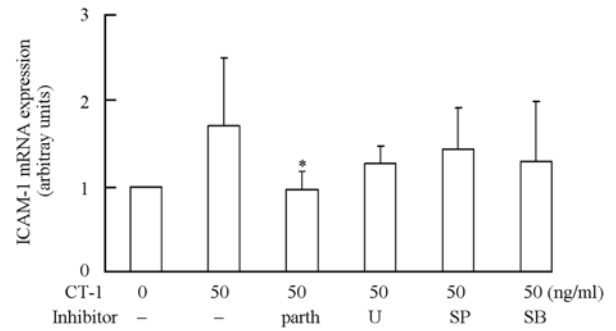


Figure 3. Confluent HUVEC were incubated with parthenolide (parth, 50 μM, NFκB inhibitor), U0126 (U, 50 μM, MEK inhibitor), SP600125 (SP, 10 μmol/L, JNK inhibitor) and SB203580 (SB, 5 μmol/L, p38 inhibitor). After 30 minutes CT-1 (50 ng/ml) was added for additional 6 hours (0: control). RNA was isolated and ICAM-1 mRNA was determined by real-time-PCR. *n*=6, data are expressed as mean ± SD. **P* < 0.05 (CT-1 50 ng/ml compared to inhibitor).

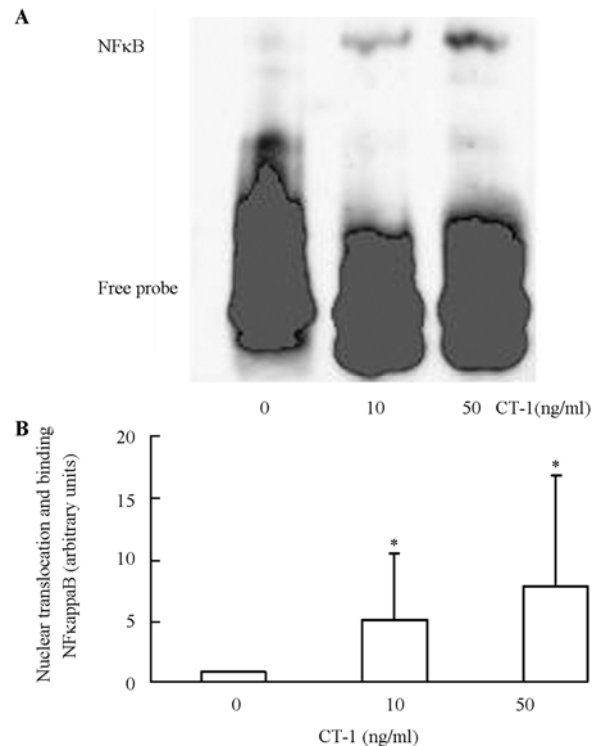


Figure 4. CT-1 induced NFκB activation. (A) Confluent layers of HUVEC were exposed to CT-1 at various concentrations for 25 minutes. Afterwards nuclear protein extracts were harvested and EMSA was performed. (B) Quantitative intensities of NFκB bands shown in figure 4B normalized to control. *n*=8, data are expressed as mean ± SD. **P* < 0.05.

CHF have changed from a hemodynamical view to a multisystem disease. Now it is common sense that nearly all clinical signs of CHF can be induced by inflammatory cytokines. Cytokines are small peptides that modulate cellular interactions. They are involved in many biological processes and they promote inflammation, intravascular coagulation, radical generation, endothelial injury and apoptosis of cardiomyocytes and endothelial cells.^{31,32} Cytokines are produced by immunological cells and cells of the cardiovascular system.^{21,33} The best studied proinflammatory cytokines are interleukin-1,

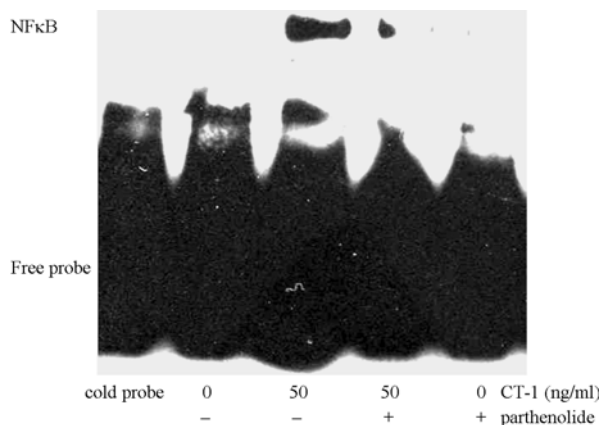


Figure 5. Representative EMSA showing the effect of parthenolide on NFκB translocation. Confluent layers of HUVEC were exposed to CT-1 and parthenolide for 25 minutes. Afterwards nuclear protein extracts were harvested and EMSA was performed.

interleukin-6 and tumor necrosis factor α . These cytokines contribute to the syndrom of CHF³⁴ and to the underlying cardiomyopathic process of adverse left ventricular remodeling and of progressive left ventricular dysfunction.^{35,36} Beside proinflammatory cytokines also adhesion molecules are found increased in sera of patients suffering from CHF.³⁷ There are a lot of studies which investigated the role of ICAM-1 or the soluble form of ICAM-1 (sICAM-1) in CHF. Devaux et al²⁷ could show that in failing human hearts adhesion molecules (for example ICAM-1) are upregulated independently of the origin of CHF indicating a low grade inflammation. In another study Wilhemi et al³⁸ found that endothelial upregulation of adhesion molecules is predominantly seen in myocardial microvasculature. Adhesion molecules are not only found increased in myocardial biopsies but also in the plasma. This could be shown by Tousoulis et al³⁹ who reported that plasma vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 levels were increased in heart failure independently of angiographically obvious atherosclerotic coronary artery disease. Furthermore Noutsias could show that in dilatative cardiomyopathy sICAM-1 correlates with myocardial ICAM-1 expression in endomyocardial biopsies.⁴⁰ ICAM-1 serum concentration increases with New York Heart Association class in CHF.²⁹ In this study sP-selectin could provide prognostic information. Treatment of heart failure decreases elevated ICAM-1 concentrations as was shown by Parissis and Wang et al.^{41,42}

In the light of these studies our data indicate that CT-1, a cytokine increased in CHF, is able to activate – measured by ICAM-1 expression – endothelial cells *in vitro*. We could show that CT-1 not only induces ICAM-1 mRNA and protein but also increases the soluble fraction of the ICAM-1 molecule. Because in the adult the heart is the main source of CT-1 we speculate that in CHF CT-1 may be at least in part responsible for increased ICAM-1 expression in the microvasculature. Furthermore the fact that CT-1 is increased independently of the origin of CHF

may explain that increased ICAM-1 expression is also independent of the pathogenesis of CHF. Ichiki et al⁴³ showed that in human aortic endothelial cells CT-1 is able to induce ICAM-1 mRNA and protein expression in the same range we found in HUVEC.

We used relatively high CT-1 concentrations in our experiments to detect endothelial cell activation compared to serum concentrations published for healthy subjects or patients with CHF. But so far the exact concentration of CT-1 in the myocardium is not reported. Because CT-1 is mainly produced in the heart in CHF,⁴⁴ we speculate that the intracoronary CT-1 concentration is much higher than CT-1 concentrations measured in venous serum. Furthermore our CT-1 concentrations are in the range Ichiki et al⁴³ used. On the other hand a recently published study showed CT-1 serum concentrations of about 100 ng/ml in control persons and patients with metabolic syndrome.⁴⁵ The discrepancy in CT-1 serum concentrations between different studies may be explained by different methods. So far the physiological CT-1 serum concentration remains to be determined.

ICAM-1 is expressed constitutively on the surface of various cell types, for example fibroblasts, leukocytes, endothelial and epithelial cells, and is up-regulated by a number of inflammatory mediators and proinflammatory cytokines.³⁰ Furthermore the level of ICAM-1 surface expression depends on the concentration of pro- and antiinflammatory mediators and on the availability of specific receptor mediated signal transduction pathways and their transcription factor targets on the ICAM-1 promotor.⁴⁶ The most important transcription factor in ICAM-1 regulation is NFκB but also the mitogen-activated protein kinases ERK, JNK and p38 are involved in some cells. Based on this we examined the pathway responsible for CT-1 induced ICAM-1 expression. Our data show that CT-1 is not able to phosphorylate ERK, JNK and p38 indicating that MAP kinases are not involved in CT-1 signaling in HUVEC. In our experiments only NFκB is responsible for ICAM-1 induction. Zhou et al reported that TNF like CT-1 induced ICAM-1 in HUVEC and that this induction is solely NFκB dependent in HUVEC.⁴⁷ In opposite, Zhou could also show an activation of the MAK-kinases after TNF stimulation which could not be observed after CT-1 stimulation. Our data are in part somewhat different as the data reported by Ichiki et al.⁴³ This group described not only the activation of NF κB but also an activation of ERK1/2, p38 and phosphatidylinositol 3-kinase. This difference may be explained by the different cells systems which were used.

In conclusion, this study demonstrates that at least *in vitro* CT-1 is able to induce ICAM-1 in HUVEC. ICAM-1 expression is mediated exclusively by the NFκB pathway and not by activation of ERK, JNK and p38.

Acknowledgements: We are grateful to Annett Schmidt and colleagues for their excellent technical assistance.

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(Received May 28, 2008)

Edited by WANG Mou-yue and LIU Huan