## Original Article

# Bilirubin effect on endothelial adhesion molecules expression is mediated by the NF-κB signaling pathway

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| Summary | We have recently demonstrated that unconjugated bilirubin (UCB) limits the over-expression of adhesion molecules and inhibits the PMN endothelial adhesion induced by the pro-inflammatory cytokine TNFα. To understand the molecular events involved we investigated whether the inhibitory effect is determined by a direct influence of UCB on different nuclear pathways. Co-treatment of cells with UCB, TNFα, and pyrridoline dithiocarbamate (PDTC), a NF-κB inhibitor, additively enhanced the inhibitory effect of UCB. UCB prevented the nuclear translocation of NF-κB induced by TNFα. The failure of UCB to alter TNFα-induced phosphorylation of cAMP-response element-binding protein (CREB) suggested that the CREB pathway is not involved in the UCB inhibition and that UCB blunting effect on the overexpression of adhesion molecules occurs *via* inhibition of the NF-κB transduction pathway. Collectively these data may contribute to explain the protective effect of bilirubin against development of atherosclerosis. |
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***Keywords:*** Endothelial cell activation, bilirubin, adhesion molecules, NF-κB, TNFα, atherosclerosis

## 1. Introduction

The earliest events in the development of atherosclerosis involve progressive modifications in the endothelial micro-environment. This endothelial cell activation, a complex of multi-step mechanisms also characterized by increased expression of adhesion molecules, mediates the diapedesis (migration) of inflammatory and immunocompetent cells through the endothelial layer into the arterial wall. The over-expression of adhesion molecules is orchestrated by pro-inflammatory cytokines, particularly TNFα (*1,2*). The two major subsets of adhesion molecules participating in these processes are the selectins (in particular E-selectin) and the immunoglobulin gene superfamily (in particular

intercellular adhesion molecule 1, ICAM-1 and cell vascular adhesion molecule 1, VCAM-1).

In different endothelial cell models, the induction of adhesion molecules expression by TNFα is triggered by two different transcriptional factors, NF-κB and cAMP-response element (CRE)-binding protein (CREB). NF-κB is a ubiquitously expressed family of transcription factors controlling inflammatory and immune responses (*3*). The most abundant form of NF-κB is an heterodimer of p50 and p65; NF-κB is sequestered in the cytoplasm in an inactive form through interaction with the IkB inhibitor proteins (*4*). Signals that induce NF-κB release dimers to enter into the nucleus and induce gene expression (*5*). A metal-chelating compound, pyrridoline dithiocarbamate (PDTC), inhibits NF-κB by blocking ubiquitine ligase activity towards phosphorylated Iκ-B (*6*), in turn downregulating the expression of E-selectin, ICAM-1 and VCAM-1 (*7*). CREB is a widely expressed DNA-binding protein and a downstream target of cAMP. CREB is activated by phosphorylation on serine 133

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(*8*). A regulatory site, on the gene promoters of both E-selectin and VCAM-1, binds both NF-κB and CREB transcription factors (*9,10*).

Unconjugated bilirubin (UCB), long considered to be simply a waste end product of heme metabolism and a marker for hepatobiliary disorders, is now thought to function as an endogenous tissue protector by attenuating free radical-mediated damage to both lipids and proteins (*11*). There is increasing epidemiological evidence supporting an inverse association between cardiovascular disease and plasma levels of bilirubin (*12*). We recently demonstrated that UCB, at clinically relevant concentrations, limits the over-expression of adhesion molecules and inhibits the PMN endothelial adhesion induced by the pro-inflammatory cytokine TNFα, even though UCB itself does not alter expression of these adhesion molecules (*13*). These results support the concept that modestly elevated concentrations of UCB, as in Gilbert's syndrome (*14*), may help prevent atherosclerotic disease, as suggested by epidemiological studies. The aim of this study is to investigate the effect of UCB on the transcription factors that regulate the surface expression of adhesion molecules.

## 2. Materials and Methods

### 2.1. *Materials*

Dulbecco's phosphate saline (DPS), Dulbecco's modified Eagles's medium high glucose (DME/HIGH), penicillin, and streptomycin were purchased from Euroclone, UK. Fetal calf serum was obtained from Invitrogen, Carlsbad, CA, USA. Chloroform (HPLC grade, 99%) was obtained from Carlo Erba, Milan, Italy. Fatty acid free bovine serum albumin (BSA), DMSO (HPLC grade), UCB (Sigma Chemical Co., St. Louis, MO, USA), TNFα, and all other reagents and chemicals were purchased from Sigma-Aldrich, Milan, Italy.

### 2.2. *Cell cultures*

H5V cells, murine heart endothelial immortalized cells (*15*) (kindly gifted by "*Istituto Mario Negri*", Milan, Italy), were grown to 80% confluence in DME/HIGH containing fetal calf serum 10% (v/v) and penicillin/ streptomycin 100 U/mL/100 μg/mL. When confluence was achieved, cells were washed three times with PBS and incubated as described in details below.

### 2.3. *Studies of the cellular effects of UCB and cytokines*

UCB was purified as described by McDonagh and Assisi (*16*) and dissolved in DMSO (0.3 μL of DMSO *per* μg of UCB, and diluted with 21 mL of serum free medium containing 30 μM bovine serum albumin (BSA). Experiments were performed at unbound UCB concentrations (Bf) of 15 and 30 nM (*17*). To minimize

photodegradation, all experiments with UCB were performed under dim lighting in vials wrapped in aluminium foil.

H5V cells were incubated in serum-free medium (DME/HIGH) containing BSA (30 μM) and DMSO (0.3%, v/v) with six different combinations of adducts: A) Control group: no adducts; B) TNFα group: add TNFα 20 ng/mL; C) UCB 15: add UCB to Bf of 15 nM; D) UCB 30: add UCB to Bf of 30 nM; E) Co-treatment UCB15-TNFα: add UCB to Bf 15 nM and TNFα 20 ng/ mL; F) Co-treatment UCB30-TNFα: add UCB to Bf 30 nM and TNFα 20 ng/mL.

A 7th group of H5V cells were treated for 2 h with pyrrolidine dithiocarbamate (PDTC, 10 μM), a specific inhibitor of NF-κB, either alone or with UCB, as described above, in the presence or absence of TNFα (20 ng/mL), added 1 h after PDTC. PDTC was dissolved in serum free medium on the day of treatment. Cells were then collected and mRNA extracted and real time RT-PCR performed as described below. An "additive effect" of UCB and PDTC was concluded only when the sum of the individual inhibitions by UCB and PDTC did not differ statistically from the experimentally-measured inhibition obtained by combined treatment with UCB and PDTC (*18*).

### 2.4. *RNA isolation and real-time RT-PCR analysis*

The H5V monolayer cells were cultured on 6 well plates and pre-treated for 2 h, with different UCB concentration with or without TNFα (20 ng/mL). Total RNA was isolated using Tri Reagent solution according to the manufacture's protocol (T9424, Sigma-Aldrich, Milan, Italy). RNA samples were quantified in a spectrophotometer at 260 nm. Agarose gel electrophoresis and staining with ethidium bromide, indicated that the RNA preparations were of high integrity.

Retrotranscription using 1 μg of total RNA was performed with an iScript cDNA Synthesis Kit (BIORAD Cat. No. 170-8891) according to the manufacture's suggestions. The reaction was run in a thermocycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) at 25°C for 5 min, 42°C for 45 min, 85°C for 5 min. The final cDNA was conserved at –20°C until used.

Real-time PCR was performed according to the iQ SYBER Green Supermix (Bio-Rad, Hercules, CA, USA) protocol. PCR amplification was carried out in 25 μL reaction volume containing 25 ng of cDNA, 1× iQ SYBR Green Supermix (100 mM KCl; 40 mM Tris-HCl, pH 8.4; 0.4 mM each dNTP; 50 U/mL iTaq DNA polymerase; 6 mM $MgCl_2$; SYBR Green I; 20 mM fluorescein; and stabilizers) (Bio-Rad Laboratories) and 250 nM gene specific sense and anti-sense primers. The selected host genes and their primer sequences were specific for the detection of E-selectin, ICAM-1, VCAM-1, β-actin, as described previously (*13*). The primers were designed using Beacon Designer 2.0

software (PREMIER Biosoft International, Palo Alto, CA, USA) choosing specific sequences crossing two contiguous exons. Reactions were run and analyzed on a Bio-Rad iCycler iQ Real-Time PCR detection system (iCycler IQ software, version 3.1; Bio-Rad). Cycling parameters were determined, and resulting data were analyzed by using the comparative $C_t$ method as the means of relative quantitation (*19*), normalized to the housekeeping gene and expressed as $2^{-\Delta\Delta C_T}$. Melting curve analysis and gel electrophoresis were performed to asses product specificity.

### 2.5. *Western blot analyses*

After pre-treatment as described above for 30 min, with different UCB concentrations, with or without TNFα (20 ng/mL), cells were washed once with PBS at room temperature and dissolved in cell lysis buffer [PBS containing 1% (v/v) of a protease inhibitor cocktail from Sigma (P-8340) and 2 mM phenylmethyl-sulfonylfluoride]. Cells were then placed on ice and disrupted by sonication (Bandelin Sonoplus, HD2070, Berlin, Germany) 3 times for 5 sec at 30% of power). Protein concentration in the lysate was determined by bicinchonic acid protein assay (BCA) following the manufacturer's instructions (B-9643, Sigma). Samples were immersed in a boiling water bath for 5 min and then immediately settled on ice. Proteins in 20 μg of cell protein lysate were size-separated, together with molecular weight standards (Precision Plus Protein dual colour standards, Bio-Rad), by SDS-PAGE on 10% polyacrylamide gel, using a Mini Protein III Cell (Bio-Rad). After SDS-PAGE, proteins were electro transferred with a semi-dry blotting system at 100 V for 120 min onto immune-blot PVDF membranes (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were incubated overnight at 4°C with commercial antibodies that allows the specific recognition of NF-κB and phosphorylated CREB at Ser 133. Antibodies were dissolved in a solution containing skim milk (5%) and T-TBS buffer (20 mM Tris, 0.2% Tween 20, 500 nM NaCl, pH 7.5). To assay NF-κB, we used a commercial antibody specific for p65 subunit (SC-109, dilution 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA,). The nuclear enrichment was proved by identification of the nuclear matrix protein p84 using a specific monoclonal antibody (ab487, dilution 1:1,000), Abcam, Cambridge, UK). Secondary antibodies conjugated with peroxidase (both from Sigma-Aldrich) IgG-anti-rabbit (dilution 1:2,000), for NF-κB, and IgG-anti-mouse (dilution 1:2,000), for p84, were used.

The phosphorylated CREB at Ser 133 was detected by a phosphor-CREB antibody (9190, Cell Signaling) at a dilution of 1:500 (recognized only phosphorylated form). The membranes were reprobed with an antibody against total CREB (recognized phosphorylated and non phosphorylated form) at a dilution of 1:500.

Both antibodies were analysed by the same procedure previously described (*13*).

The peroxidase reaction was obtained by exposure of membrane in the ECL-Plus Western Blot detection system solutions (ECL Plus Western Blotting Detection Reagents, GE-Healthcare Bio-Sciences, Italia). Autoradiographic band intensities were estimated by densitometric scanning using NIH Image software (Scion Corporation, Frederick, MD, USA).

### 2.6. *Preparation of total nuclear extracts after UCB treatment*

The total cytoplasmic and nuclear extracts were obtained by Digman's method (*20*) with minor modification. $5 \times 10^7$ H5V cells were seeded in 75-cm$^2$ flasks and were treated with different UCB concentrations with or without TNFα (20 ng/mL) for 30 min. After treatment, the cells were collected by centrifugation at 800 × g for 10 min. The cells were resuspended in 400 μL ice-cooled solution A (10 mM Hepes, pH 7.9, 0.1 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, pH 8, 0.1 mM dithiotreitol, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM Na orthovanadate, and 1 mM Na fluoride). After 10 min incubation on ice, the cells were centrifuged at 800 × g for 5 min at 4°C. The supernatant containing the cytoplasm was collected and stored at –80°C. The pellet containing nuclei was resuspended with solution A and was centrifuged at 800 × g for 5 min at 4°C. The nuclear fraction was resuspended in ice-cooled solution B (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5% (v/v) glycerol, 0.1 mM dithiotreitol, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM Na orthovanadate, and 1 mM Na fluoride). After 30 min incubation on ice with constant stirring, the suspension was vortexed for 10 sec, then centrifuged at 15,000 × g for 20 min at 4°C. The supernatant containing nuclear extract was recovered and stored at –80°C. The protein content of the extracts was determined by BCA method.

### 2.7. *Statistical analysis*

All experiments were run in triplicate and repeated three times. Results are expressed as mean ± S.D. One way ANOVA with Tukey-Kramer post test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). Probabilities < 0.05 were considered statistically significant.

### 3. Results

### 3.1. *Effect of PDTC pretreatment on TNFα-induction of adhesion molecule mRNA expression in H5V cells*

For all three adhesion molecules investigated (E-selectin, VCAM-1, and ICAM-1), pretreatment with 10 μM PDTC

significantly inhibited the gene over-expression induced by TNFα ($p < 0.05$) (Figure 1). A further inhibition of gene expression by co-treatment with UCB was seen for: E-Selectin at Bf of 15 and 30 nM and of VCAM-1 but only at a Bf of 30 nM. ICAM-1 gene induction by TNFα was not further inhibited by the addition of UCB to cells pre-treatment with PDTC.

### 3.2. *TNFα-induced nuclear translocation of NF-κB is inhibited by UCB*

As reported, TNFα stimulated nuclear translocation of NF-κB (*4,5*), detected with antibody against its p65 subunit. UCB itself did not affect NF-κB translocation (data not shown) but inhibited the nuclear translocation of NF-κB induced by TNFα in a dose dependent manner (Figure 2). In addition, co-treatment with UCB and TNFα caused an increase in the cytoplasmic fraction of NF-κB compared to control and to TNFα alone treatment.

### 3.3. *Phosphorylation of cAMP-response element (CRE)-binding protein (CREB) is influenced by TNFα but not UCB.*

Phosphorylation of CREB was significantly increased in a time dependent manner by TNFα alone, with a
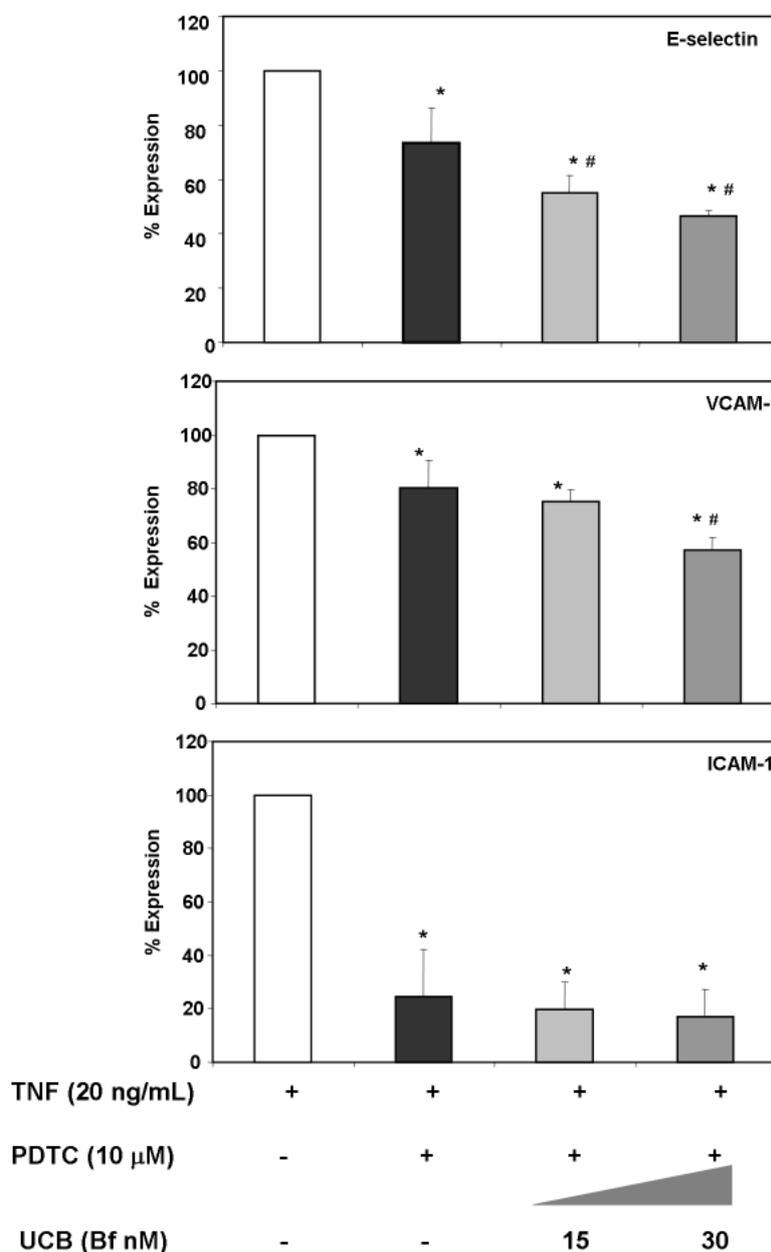


**Figure 1. UCB and pyrridoline dithiocarbamate (PDTC) additively inhibit the overexpression of adhesion molecule mRNA induced by TNFα.** Effect of UCB (Bf = 15 and 30 nM), with or without TNFα (20 ng/mL) and/or PDTC (10 μM) on H5V cells. Cells were collected after 2 h and the mRNA was analyzed by real time RT-PCR. Results are expressed as percent expression (mean ± SD) of 3 experiments, related to treatment with TNFα alone, considered as 100% (unshaded bar). *: $p < 0.05$ *versus* treatment with TNFα alone. #: $p < 0.05$ *versus* TNFα and PDTC treatments. See text for details.
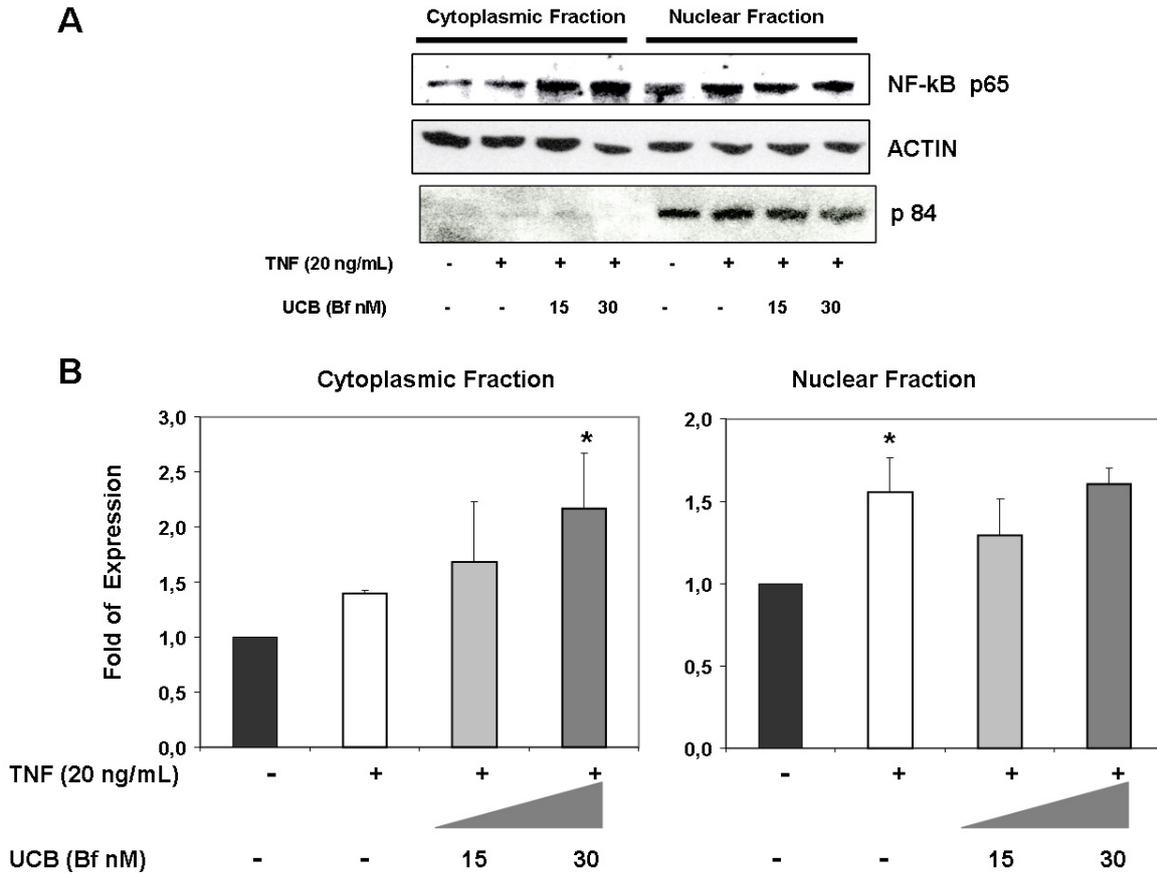
**Figure 2. UCB inhibits TNFα-induced nuclear translocation of NF-κB in H5V cells.** (**A**) Western blots after 30 min of incubation with TNFα and/or UCB. NF-κB was detected using a p65 NF-κB antibody. The purity of the cytoplasmic fraction of NF-κB was confirmed by anti-P84 antibody. TNFα stimulated translocation of NF-κB from cytoplasm to nucleus, which was inhibited by UCB. (**B**) The density of each specific band was scanned and quantified with an imaging analyzer, and normalized by actin. The bars show the normalized densities (mean ± SD of three reproducible experiments) relative to the cytoplasmic and nuclear fractions of the non stimulated cells, set at 1.0. *: $p < 0.05$ *versus* non stimulated controls.
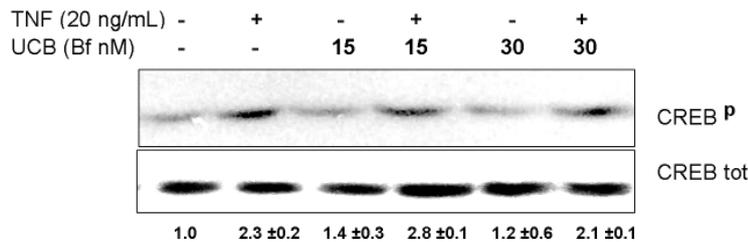


**Figure 3. UCB does not affect CREB phosphorylation in H5V cells.** TNFα induced CREB phosphorylation at Ser 133. UCB, either alone or in cotreatment with TNFα did not affect CREB phosphorylation. Phosphorylation of CREB was detected by Western blot analysis of cell lysate obtained after 15 min of incubation with TNFα and/or UCB, using an antibody that recognizes the form of CREB phosphorylated at Ser 133 and an antibody that recognizes both forms of CREB. The density of the specific band was scanned and quantified with an imaging analyzer. The numbers show the normalized ratio of phosphorylated CREB to total CREB (mean ± SD of three reproducible experiments) in TNFα stimulated cells, relative to the ratio in the unstimulated cells, set at 1.0.

maximum reached after 15 min (data not shown). UCB alone or in co-treatment with TNFα did not affect CREB phosphorylation (Figure 3).

**4. Discussion**

For a long time bilirubin was considered to be simply a waste end product of heme metabolism. More recently evidence has emerged pointing to bilirubin as an independent factor in the prevention of atherosclerotic disease (*21*). In particular, mildly elevated serum bilirubin levels were associated with a lower incidence of ischemic cardiovascular effects (*14*) raising the idea that UCB can interfere with the mechanisms involved in the development of atherosclerosis. Based on the antioxidant properties of bilirubin, an hypothesis was formulated that UCB acts as a scavenger of reactive oxygen species (ROS) (*22*). We recently demonstrated

that unbound bilirubin at a concentration, similar to the plasma Bf levels found in humans with mild unconjugated hyperbilirubinemia (Bf, 15 and 30 nM) (*23,24*) and therefore clinically relevant, blunts the over-expression of E-selectin and VCAM-1 mRNA induced by TNFα (*13*).

Several signalling pathways are involved in regulating the gene expression of these adhesion molecules, especially NF-κB (*25,26*) and CREB (*10,27*). E-selectin, ICAM-1, and VCAM-1 are known to share many common regulatory mechanisms, but only partially the NF-κB signal transduction pathway (*7,28*), for which UCB is known to be a modulator (*29*). In H5V cells, the TNFα-stimulated nuclear translocation of NF-κB was inhibited by UCB, confirming that UCB can affect the NF-κB regulatory pathway, probably through an interaction with IKK proteins (*30*).

We found that PDTC, an IkBα inhibitor that prevents the release of p65 NF-κB (*31*), has an additive effect on the UCB- induced inhibition of TNFα-induction of adhesion molecules. This finding indicates that bilirubin may act through NF-κB signalling cascade. Although CREB is involved in the upregulation of VCAM-1 and E-selectin gene expression induced by TNFα (*10,27*), we did not observe any influence of UCB on the phosphorylation of CREB induced by TNFα. Thus, CREB probably does not mediate the influence of UCB on the expression of the adhesion molecules in H5V cells.

In summary, our data indicate that UCB limits the over-expression of adhesion molecules and inhibits the PMN endothelial adhesion induced by the pro-inflammatory cytokine TNFα, even though UCB itself does not alter expression of these adhesion molecules (*13*). This effect is in part mediated by modulation of the NF-κB transcription factor. These results provide molecular support to the concept that modestly elevated concentrations of UCB, as in Gilbert's syndrome (*14*), may help prevent atherosclerotic disease, as suggested by epidemiological studies.

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**References**

1. Kelly M, Hwang JM, Kubes P. Modulating leukocyte recruitment in inflammation. J Allergy Clin Immunol. 2007; 120:3-10.

2. Young JL, Libby P, Schonbeck U. Cytokines in the pathogenesis of atherosclerosis. Thromb Haemost. 2002; 88:554-567.

3. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell. 2002; 109 (Suppl):S81-S96.

4. Baeuerle PA. Pro-inflammatory signaling: last pieces in the NF-kappaB puzzle? Curr Biol. 1998; 8:R19-R22.

5. May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today. 1998; 19:80-88.

6. Traenckner EB, Wilk S, Baeuerle PA. A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. EMBO J. 1994; 13:5433-5441.

7. Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest. 1993; 92:1866-1874.

8. Wang AB, Li HL, Zhang R, She ZG, Chen HZ, Huang Y, Liu DP, Liang CC. A20 attenuates vascular smooth muscle cell proliferation and migration through blocking PI3k/Akt singling *in vitro* and *in vivo*. J Biomed Sci. 2007; 14:357-371.

9. Edelstein LC, Pan A, Collins T. Chromatin modification and the endothelial-specific activation of the E-selectin gene. J Biol Chem. 2005; 280:11192-11202.

10. Ono H, Ichiki T, Ohtsubo H, Fukuyama K, Imayama I, Iino N, Masuda S, Hashiguchi Y, Takeshita A, Sunagawa K. CAMP-response element-binding protein mediates tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in endothelial cells. Hypertens Res. 2006; 29:39-47.

11. Neuzil J, Stocker R. Bilirubin attenuates radical-mediated damage to serum albumin. FEBS Lett. 1993; 331:281-284.

12. Rigato I, Ostrow JD, Tiribelli C. Bilirubin and the risk of common non-hepatic diseases. Trends Mol Med. 2005; 11:277-283.

13. Mazzone GL, Rigato I, Ostrow JD, Bossi F, Bortoluzzi A, Sukowati CH, Tedesco F, Tiribelli C. Bilirubin inhibits the TNFα-related induction of three endothelial adhesion molecules. Biochem Biophys Res Commun. 2009; 386:338-344.

14. Vitek L, Jirsa M, Brodanova M, Kalab M, Marecek Z, Danzig V, Novotny L, Kotal P. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. Atherosclerosis. 2002; 160:449-456.

15. Garlanda C, Parravicini C, Sironi M, De Rossi M, Wainstok DC, Carozzi F, Bussolino F, Colotta F, Mantovani A, Vecchi A. Progressive growth in immunodeficient mice and host cell recruitment by mouse endothelial cells transformed by polyoma middle-sized T antigen: implications for the pathogenesis of opportunistic vascular tumors. Proc Natl Acad Sci U S A. 1994; 91:7291-7295.

16. McDonagh AF, Assisi F. The ready isomerization of bilirubin IX- in aqueous solution. Biochem J. 1972; 129:797-800.

17. Roca L, Calligaris S, Wennberg RP, Ahlfors CE, Malik SG, Ostrow JD, Tiribelli C. Factors affecting the binding of bilirubin to serum albumins: validation and application of the peroxidase method. Pediatr Res. 2006; 60:724-728.

18.  Kuldo JM, Westra J, Asgeirsdottir SA, Kok RJ, Oosterhuis K, Rots MG, Schouten JP, Limburg PC, Molema G. Differential effects of NF-{kappa}B and p38 MAPK inhibitors and combinations thereof on TNF-{alpha}- and IL-1{beta}-induced proinflammatory status of endothelial cells *in vitro*. Am J Physiol Cell Physiol. 2005; 289:C1229-C1239.

19.  Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45.

20.  Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 1983; 11:1475-1489.

21.  Djousse L, Levy D, Cupples LA, Evans JC, D'Agostino RB, Ellison RC. Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. Am J Cardiol. 2001; 87:1196-1200.

22.  Stocker R, Keaney Jr JF. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004; 84:1381-1478.

23.  Jacobsen J, Wennberg RP. Determination of unbound bilirubin in the serum of newborns. Clin Chem. 1974; 20:783.

24.  Nelson T, Jacobsen J, Wennberg RP. Effect of pH on the interaction of bilirubin with albumin and tissue culture cells. Pediatr Res. 1974; 8:963-967.

25.  Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 2002; 13:413-421.

26.  Lin CW, Chen LJ, Lee PL, Lee CI, Lin JC, Chiu JJ. The inhibition of TNF-alpha-induced E-selectin expression in endothelial cells *via* the JNK/NF-kappaB pathways by highly *N*-acetylated chitooligosaccharides. Biomaterials. 2007; 28:1355-1366.

27.  Gerritsen ME, Williams AJ, Neish AS, Moore S, Shi Y, Collins T. CREB-binding protein/p300 are transcriptional coactivators of p65. Proc Natl Acad Sci U S A. 1997; 94:2927-2932.

28.  Zerfaoui M, Suzuki Y, Naura AS, Hans CP, Nichols C, Boulares AH. Nuclear translocation of p65 NF-kappaB is sufficient for VCAM-1, but not ICAM-1, expression in TNF-stimulated smooth muscle cells: Differential requirement for PARP-1 expression and interaction. Cell Signal. 2008; 20:186-194.

29.  Fernandes A, Falcao AS, Silva RF, Gordo AC, Gama MJ, Brito MA, Brites D. Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. J Neurochem. 2006; 96:1667-1679.

30.  Malek S, Chen Y, Huxford T, Ghosh G. IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. J Biol Chem. 2001; 276:45225-45235.

31.  Schoonbroodt S, Piette J. Oxidative stress interference with the nuclear factor-kappa B activation pathways. Biochem Pharmacol. 2000; 60:1075-1083.