Original Article

Unconjugated bilirubin modulates nitric oxide production *via* iNOS regulation

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Summary **To induce the *in vitro* endothelial dysfunction model, H5V cells were treated with tumor necrosis factor α (TNFα) and with unconjugated bilirubin (UCB) at two different physiological concentrations. The TNFα-induced reduction of nitric oxide (NO) concentration was reversed by UCB. Endothelial NO synthase (eNOS) gene expression was not influenced by treatments while inducible NO synthase (iNOS) expression was increased at 24 h. Co-treatment of H5V cells with pyrrolidine dithiocarbamate, TNFα (20 ng/mL) and UCB (Bf 15 or 30 nM) for 2 h caused a significant reduction of iNOS gene expression. We conclude that at physiological concentrations UCB prevents endothelial dysfunction by modulating NO concentration probably through inhibition of NF-κB.**

Keywords: Bilirubin, nitric oxide (NO), endothelial dysfunction

1. Introduction

Nitric oxide (NO) is one of the pivotal factors involved in the prevention of atherosclerotic lesions of the endothelium. It has been demonstrated that NO plays a critical role also in vascular hyper-permeability through both NO synthases (NOSs) expression, the endothelial form (eNOS) and an the inducible one (iNOS) *in vitro* (*1*) together with down-regulation of cytokine-induced endothelial cell adhesion molecule expression (*2*). Conversely, the inhibition of the NO producing enzyme eNOS causes an accelerated atherosclerotic process in experimental models (*3*).

Most probably eNOS has a double role in the pathogenesis of atherosclerosis. Under normal conditions, eNOS generates low concentrations of NO and probably peroxynitrite (*4*), favoring an anti-atherosclerotic environment (*5,6*). However, during hyperlipidemia and atherosclerosis, it may contribute to the formation of oxidative stress by a reduction in BH4-dependent NO formation and unopposed superoxide formation (*7*). In particular, in the setting of local induction, iNOS could favour the development of local toxic concentrations of peroxynitrite in atherosclerotic plaques (*8*).

Bilirubin, and in particular its unbound active form (unconjugated bilirubin; UCB), has been suggested to act as an endogenous tissue protector by attenuating radical-mediated damage to both lipids and proteins (*9*). There is increasing epidemiological evidence supporting an inverse association between cardiovascular disease and plasma levels of bilirubin (*10,11*). Recently we demonstrated that bilirubin may be protective in the development of atherosclerotic diseases by blunting the expression of E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (*12*) through a regulation of the NF-κB pathway (*13*). The earliest event that occurs in the development of atherosclerosis is characterized by a progressive modification in the physiological micro-environment identified as endothelial dysfunction (*14*). Endothelial dysfunction is a complex, multi-step mechanism where reduced NO levels have been reported as a marker (*5*) and, at the same time is characterized by increasing expression of adhesion molecules (AM).

Since the biological effects of UCB and the mechanisms of these effects on the development of atherosclerosis have yet to be explored, the aim of this study is to investigate the effect of UCB on NO production.

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2. Materials and Methods

2.1. *Materials*

Dulbecco's modified Eagles's medium high glucose (DMEM/HIGH), penicillin, and streptomycin were purchased from EuroClone, Siziano, Italy. 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), dimethyl sulfoxide (DMSO, HPLC grade), UCB, tumor necrosis factor α (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*) (kind gift from Istituto Mario Negri, Milan, Italy), were grown to 80% confluence in DMEM/HIGH containing 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. When confluence was achieved, cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) and incubated as described 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 DMSO *per* μg UCB, and diluted with 21 mL serum free medium containing 30 μM 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 (DMEM/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; and F) co-treatment UCB 30-TNFα, add UCB to Bf 30 nM and TNFα 20 ng/mL.

A further group of H5V cells were treated for 2 h with pyrrolidine dithiocarbamate (PDTC, 10 μM), a specific inhibitor of NF-κB (*18*), 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 previously described (*12*).

2.4. *Mitochondrial toxicity determined by MTT test*

A stock solution of 3(4,5-dimethyltiazolyl-2)-2,5 diphenyl tetrazolium (MTT) was dissolved in PBS at 5 mg/mL (*19*). MTT solution was diluted to 0.5 mg/mL in DMEM/HIGH without phenol red. Cells were incubated with DMEM containing MTT for 2 h at 37°C at the end of the incubation period, the medium was replaced with 1 mL HCl containing 0.04 M isopropanol to dissolve MTT formazan crystals. Samples were then gently shaken for 2 h at 37°C. After centrifugation at 10,000 rpm for 3 min, absorbance at 570 nm was determined using a Beckman DU 640 spectrophotometer (Beckman Coulter S.p.A, Milan, Italy). Results were expressed as percentage of control cells, not exposed to UCB considered as 100% viability.

2.5. *LDH release test*

The presence of lactate dehydrogenase (LDH) in the culture supernatant and lysate cells was measured by a colorimetric assay, using the Cytotoxicity Detection Kit (LDH, Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions. The absorbance of the samples at 490 nm was determined in a Beckman DU 640 spectrophotometer. Results were expressed as percentage of total LDH released *vs.* control cells, not exposed to UCB.

2.6. *Analysis of nitrite ion (NO^{2-}) levels*

Colorimetric assays with Griess reagent were used to detect NO levels. Culture media were saved and absorbance was determined in a spectrophotometer at 540 nm, values were compared against a standard curve with increasing concentrations of nitrite. Results were expressed as mean percentage values (%) of control cell group.

2.7. *RNA isolation and real-time RT-PCR analysis*

H5V monolayer cells were cultured on 6 well plates and pre-treated for 2 h with different UCB concentrations with or without TNFα (20 ng/mL). RT-PCR was performed as previously described (*12*). Briefly, total RNA was isolated using Tri Reagent solution (T9424, Sigma-Aldrich, Milan, Italy) and was quantified using a spectrophotometer at 260 nm. Retrotranscription was performed with an iScript cDNA Synthesis Kit (BioRad, Cat. No. 170-8891), 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, and 85°C for 5 min. Real-time PCR was performed according to the iQ SYBER Green Supermix (Bio-Rad) protocol. The selected host genes and their primer sequences (Table 1) were designed using Beacon Designer 2.0 software (PREMIER Biosoft International, Palo Alto, CA, USA). Reactions were run and analyzed on a Bio-Rad iCycler iQ Real-Time PCR detection system (iCycler IQ software, version 3.1; Bio-Rad).

Table 1. List of primer sequences

Murine Gene	Primer Foward	Primer Reverse
eNOS	GTGGAACAACTGGAGAAAGG	AAGGAGGCGAGGACTAGG
iNOS	TTGTGCGAAGTGTCAGTGG	TCCTTTGAGCCCTTTGTGC
β-Actin	CCTTCTTGGGTATGGAATCCTGTG	CAGCACTGTGTTGGCATAGAGG

Cycling parameters were determined, and resulting data were analyzed using the comparative Ct method as the means of relative quantitation, normalized to the housekeeping gene and expressed as $2^{-\Delta\Delta Ct}$. Melting curve analysis and gel electrophoresis was performed to assess product specificity.

2.8. *Statistical analysis*

All experiments were performed in triplicate and repeated at least three times. Results are expressed as means ± 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 and Discussion

3.1. *Effects of UCB on H5V cell viability*

The effect of UCB and TNFα on endothelial cell viability was evaluated by LDH release and mitochondrial toxicity determined by MTT assay. Plasma membrane integrity, investigated through LDH release, was unaffected by different doses of UCB (15 and 30 nM free bilirubin). As previously described (*20*), the addition of TNFα (20 ng/mL) significantly increased the extracellular LDH activity. However, no further effects were observed when co-treatment with TNFα and UCB were performed (Table 2).

On the contrary, UCB reduced, in a dose dependent manner, mitochondrial function (Figure 1) assessed by MTT assay indicating that the UCB effect was mainly due to a metabolic dysfunction but was not associated with increased cellular permeability or necrosis.

3.2. *NO concentration after treatment with TNFα alone or plus UCB*

We measured nitrite production after 24 and 48 h treatment with UCB and/or TNFα (20 ng/mL) (Figures 2A and 2B, respectively). The significant reductions of nitrite content in the cell supernatants induced by TNFα were not reversed by UCB after treatment for 24 h (Figure 2A) while UCB significantly reduced nitrite production after 48 h (Figure 2B) at both low and high concentrations (Bf 15 and 30 nM).

The regulation of NO metabolism and the enzymes involved in its synthesis (eNOS and iNOS) during the pro-inflammatory state is controversial (*6*). Cytokines are believed to induce the production of substantial amounts of NO by increasing iNOS expression and activity during the pro-inflammatory state (*21*). However, eNOS down-regulation by TNFα, and a decreased bio-availability of NO caused by endothelial dysfunction were also reported (*22*). Our data showed a reduction of NO levels by the pro-inflammatory cytokine TNFα after 24 and 48 h treatment (Figures 2A and 2B).

Table 2. Effects of UCB and TNFα on cell viability using LDH assay

UCB (Bf, nM)	LDH release (%)	
	– TNFα	+ TNFα
Control	14.9 ± 1.60	26.8 ± 1.30*
15	15.2 ± 1.30	23.1 ± 1.90*
30	14.6 ± 1.80	23.4 ± 1.70*

Results are expressed as % LDH released, $n = 3$ of one experiment out of two. * $p < 0.05$ *versus* – TNFα.

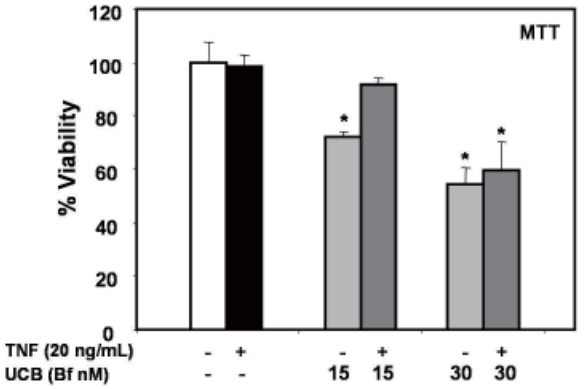

Figure 1. Effects of UCB on H5V cell viability by MTT assay. Results are expressed as mean percentage values (%) of three independent experiments performed in triplicate. Control cells (UCB, Bf 0 nM) were considered as 100%. * $p < 0.05$ *versus* controls.

3.3. *eNOS and iNOS expression after treatments with TNFα alone or plus UCB*

As shown in Figure 3, the gene expression of eNOS was not influenced by treatment with TNFα nor by different doses of UCB (15 and 30 nM) at 2, 6, and 24 h. As previously reported (*23*), TNFα increased the expression of iNOS at 2, 6, and 24 h, while no effect was observed with UCB treatment alone (data not shown). Co-treatments with TNFα and UCB indicated a slight but significant reduction of RNA expression of iNOS at 2 h while at 24 h they increased the gene expression of iNOS when compared with UCB or

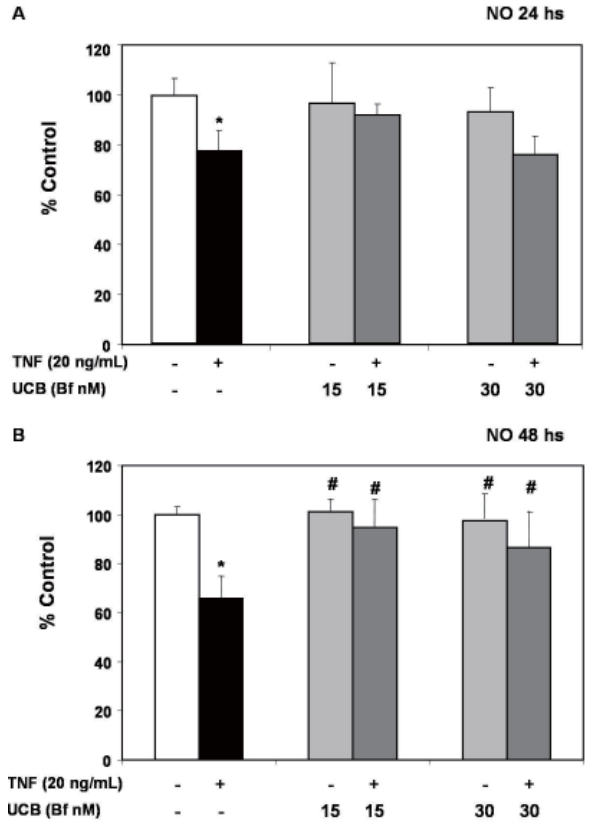

Figure 2. Effect of different doses UCB on NO production. Results are expressed as mean percentage values (%) of control cell group, from three independent experiments performed in triplicate. * $p < 0.05$ *versus* control; # $p < 0.05$ *versus* TNFα group alone (UCB, Bf 0 nM plus TNFα).

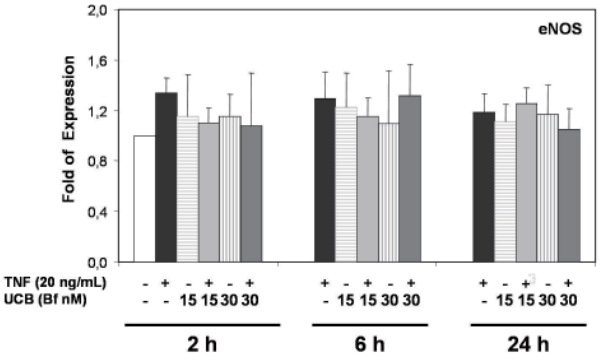

Figure 3. Effects of UCB on TNFα-induced gene expression of eNOS in H5V cells. Results are expressed as fold expression relative to respective untreated control set at 1.0.

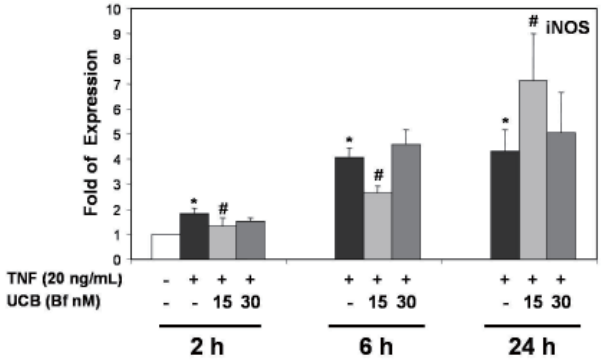

Figure 4. Effects of UCB on TNFα-induced gene expression of iNOS in H5V cells. Results are expressed as fold expression relative to respective untreated control set at 1.0. # $p < 0.05$ *versus* TNFα alone. * $p < 0.05$ *versus* controls.

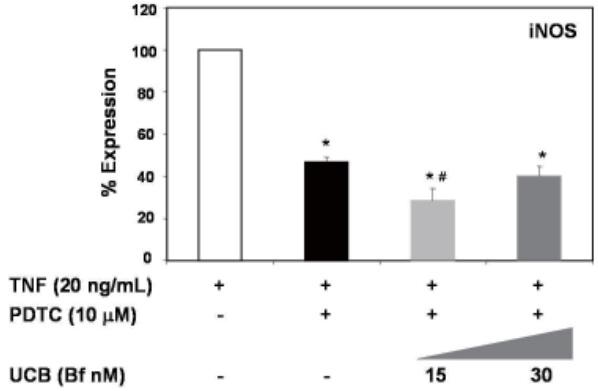

Figure 5. UCB and PDTC additively inhibit the overexpression of iNOS mRNA induced by TNFα. Results are expressed as percent expression, related to treatment with TNFα alone, considered as 100% (unshaded bar). * $p < 0.05$ *versus* treatment with TNFα alone; # $p < 0.05$ *versus* TNFα and PDTC treatments.

TNFα treatments alone (Figure 4).

Although the activity of UCB is basically related either to toxic or antioxidant effects according to its concentration (*24*), results obtained in the present and in our previous studies (*12,13*) demonstrate that UCB, even at physiological (15 nM) or mildly elevated (30 nM) concentrations, can modulate gene expression and endothelial cell function. Our data also demonstrate that UCB plays a role in the modulation of NO metabolism. The NO level is the result of a very complex regulated mechanism in which UCB may be involved at different steps. As shown in Figure 2, at 48 h, the NO level was reversed by UCB at both concentrations tested which suggests an up-regulation of iNOS induced by UCB at 24 h (Figure 4). However, UCB significantly inhibits iNOS expression at 2 h (Figure 4) when NO levels are not detectable (data not shown). This complex biphasic regulation of UCB with iNOS expression may be explained by a modulation of the NO levels. Since it was reported that NO levels are responsible for iNOS regulation itself (*25*), UCB may prevent NO induction when NO levels are not detectable. On the other hand, when NO levels are restored (at 48 h), UCB may compensate for these effects by a synergistic effect on iNOS induced TNFα (24 h). This hypothesis is further supported by the demonstration either *in vivo* and *in vitro*, that UCB limits the increase of hepatic levels of TNFα, NO, and iNOS caused by treatment with endotoxin (*26*).

Several signaling pathways, in particular NF-κB, are described as involved in regulating the gene expression of iNOS and adhesion molecules (*27*). As previously reported (*13*), UCB does not affect NF-κB translocation but inhibits the nuclear translocation of NF-κB induced

by TNFα. We found that PDTC, an IkBα inhibitor that prevents release of p65 NF-κB (*28*), has an additive effect with UCB on the inhibition of TNFα-induction of iNOS after 2 h of treatment (Figure 5).

The present results further reinforce the putative role of bilirubin in the prevention of tissue injury in response to inflammatory stimuli and, in agreement with our recent studies (*12,13*) and others on heme oxygenase-1 (*29*), indicate UCB is a potential cardiovascular protective factor.

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