

## INTER-DEPENDENCE BETWEEN CYTOKINES AND NO/NOS SYSTEM IN RESTING AND ACTIVATED ENDOTHELIAL CELLS

Vidosava B. Đorđević<sup>1</sup>, Tatjana Cvetković<sup>1</sup>, Ivana Stojanović<sup>1</sup>, Vladan Ćosić<sup>2</sup>,  
Slavica Kundalić<sup>2</sup>, Lilika Zvezdanović<sup>2</sup>, Tatjana Jevtović-Stoimenov<sup>1</sup>

<sup>1</sup>Institute of Biochemistry, University School of Medicine, Niš

<sup>2</sup>Centre of Medical Biochemistry, Clinical Centre, Niš, Serbia and Montenegro

**Summary:** Cytokines are a heterogeneous and multifunctional group of molecules synthesized in various human cells. Structurally they are peptides (often glycosylated) used by cells for intercellular communication and control the inner environment of the cells in which they operate. Cytokines are produced by the cells involved in the immune response, inflammation, hemopoiesis, healing and systemic response to injury. Immunity, inflammatory reactions and haemostasis involve close interactions between immunocompetent cells and vascular endothelium. Vascular cells are both a target for cytokines and their source. The spectrum of endothelial cell responses challenged by cytokines is wide and varied, with different cytokines activating distinct, but overlapping, sets of functions. Under normal resting conditions endothelial cells constitutively express certain protective genes with the purpose to maintain the endothelial cells in their quiescent phenotype by inhibiting NF- $\kappa$ B activation and exerting antiapoptotic functions. In this status endothelial cells can exhibit their barrier and anticoagulant functions even in the presence of low levels of stimulants. When the endothelial cells are exposed to numerous stimuli such as TNF, IL-1, endotoxin or xenoreactive antibodies and complement, which are usually associated with infections, graft rejection or autoimmune diseases such as, vasculitis, NF- $\kappa$ B induces the expression of adhesion molecules such as E-selectin, chemokines such as IL-8 and procoagulant molecules such as TF. Besides the induction of expression of a functional programme related to thrombosis and inflammation, IL-1 and TNF also induce production of autocoids including nitric oxide (NO). Both the inducible form of NO synthase (iNOS) type II and the constitutive (type III) isoform of NOS are present in endothelial cells catalyzing the conversion of arginine into citrulline and NO. The formation of NO is an ubiquitous biochemical pathway involved in the regulation of neurotransmission, vasodilatation, immunity and cytotoxicity. During inflammatory reaction NO produced by endothelial cells exerts its autocrine function through the inhibition of cytokine-induced expression of adhesion molecules and cytokine production by endothelial cells. Also, it has a protective role in inflammation through the inactivation of NADPH oxidase and the consequent impairment of superoxide production for cell mediated injury. On the other hand, there is considerable evidence that NO contributes to tissue destruction in inflammatory and immune diseases being a key component of the cytostatic/cytotoxic function of the immune system. The damage to target cells by NO released from activated macrophages or endothelial cells may involve both necrotic and apoptotic pathways of cell death.

**Key words:** endothelial cells (ECs), cytokines, nitric oxide (NO), nitric oxide synthase (NOS)

### The role of NF- $\kappa$ B in endothelial cell activation

The vascular endothelium is a specific organ weighting about two kg and representing the inner – lining of all blood vessels consistent of endothelial cells which make a unique border between the circulating

blood and the vascular wall. Because of its strategic location, the endothelium interacts with cellular and neurohumoral mediators, thus controlling vascular contractile state and cellular composition.

Endothelial cells (ECs) have long been considered as a layer of »nucleated cellophane« (passive barrier) endowed with negative properties, the most important being its ability to act as a non-thrombogenic substrate for blood. As such, endothelium was thought to participate in tissue reactions essentially as target for injurious agents. Now, it is evident that the

Address for correspondence

Prof. dr Vidosava Đorđević  
Biohemijski institut, Medicinski fakultet  
B. Tasković 81, 18000 Niš

vascular endothelium serves as an important autocrine and paracrine organ capable of maintaining vascular homeostasis by modulating blood vessel tone, by regulating local cellular growth and extracellular matrix deposition and by controlling haemostatic as well as inflammatory responses. Thus, haemostasis, inflammatory reactions and immunity involve close interactions between immunocompetent cells and vascular endothelium (1).

The activation or stimulation of ECs may be induced by various agents including cytokines, mitogens, viruses, reactive oxygen intermediates (2, 3) xeno- or allo-reactive antibodies and complement. In this process a crucial role belongs to the transcription factor, nuclear factor kappa B (NF- $\kappa$ B), multiprotein complex that can activate a great variety of genes involved in early defence reactions of higher organisms. NF- $\kappa$ B is a heterodimer composed of two subunits (p65/p105 and p50) (3, 4). Steady state levels of these transcripts are transiently increased by TNF- $\alpha$  (4). Physiologically (in non-stimulated cells) the nuclear factor, NF- $\kappa$ B exactly resides in the cytoplasm as an inactive complex bound to its specific a 60-70-kDa inhibitory protein I $\kappa$ B (5) or I $\kappa$ B- $\alpha$  (MAD-3). Protein levels of this inhibitor fall rapidly, after TNF- $\alpha$  stimulation (4). When activated, I $\kappa$ B dissociates from the NF- $\kappa$ B-I $\kappa$ B complex presumably by activation of protein kinase C (5) and NF- $\kappa$ B translocates to the nucleus, binding with high affinity to specific sites in the promoter regions of target genes and stimulating their transcription. There is evidence that mitochondrially derived reactive oxygen species play a critical role in the activation of this cytokine – sensitive transcription factor (6). The activation proceeds so that both subunits, p50 and p65 of NF- $\kappa$ B bind to, for example, the E-selectin kappa B site (4). When p50 and p65 accumulate in the nucleus RNA transcript levels for I $\kappa$ B-a are dramatically upregulated. Recombinant p65 stimulates expression of E-selectin promoter-reporter constructs while I $\kappa$ B- $\alpha$  inhibits p65 or TNF- $\alpha$ -stimulated E-selectin promoter-reporter gene expression (4). In similar way NF- $\kappa$ B induces the expression of cell adhesion molecules (ICAM-1, VCAM1 and E-selectin), cytokines, acute phase proteins, growth factors, COX-2 and iNOS (inducible nitric oxide synthase) being essentially involved in immediate early expression of various immunoregulatory genes and representing an important regulatory system of endothelial activation.

### Resting endothelial cells

The role of NF- $\kappa$ B in the induction of the proinflammatory genes in ECs activation has been shown using specific inhibitors of NF- $\kappa$ B, including its natural repressor I $\kappa$ B- $\alpha$  (7) and a truncated mutant of p65/RelA that lacks the transactivation domain (8). Also, stimulation of ECs results in upregulation of two antiapoptotic genes, encoding A1- and A20 (9), the

latter of which is dependent on NF- $\kappa$ B and can negatively regulate its own expression (10). It is possible that, under normal resting (quiescent) conditions, ECs constitutively express certain »protective« genes such as that encoding Bcl-xl (11). It has also been found that expression of A20, Bcl-2 or Bcl-xl in ECs *in vitro* inhibits activation of NF- $\kappa$ B and thus blocks induction of the proinflammatory genes (12). Bcl-2 is an intracellular membrane-associated protein that functions to block programmed cell death, but Bcl-2 protein levels are low or undetectable in ECs. A1 is the only known Bcl-2 family member that is inducible by inflammatory cytokines, suggesting that it may play a protective role during inflammation (13). It is important to mention that, besides the concept that expression of protective genes is a physiological survival response to injury, these genes are also expressed in ECs in some other situations. For example, ECs of vessels of long-surviving allografts that do not develop transplant arteriosclerosis express these some protective genes (11). It seems that the purpose of these constitutively expressed protective genes is to maintain the ECs in their quiescent phenotype by inhibiting NF- $\kappa$ B activation, as well as by exerting anti-apoptotic functions. As such, the ECs can perform their normal barrier and anticoagulant functions even in the presence of low levels of stimulants such as shear stress, circulating endotoxin or reactive oxygen species (ROS). In these instances, a proinflammatory response would presumably have no purpose.

### Endothelial cell activation

In case of endothelial cell activation, the proinflammatory response of the ECs can not be counteracted by the constitutively expressed protective genes, thereby allowing accumulation of ROS and activation of a cascade of proteases involved in the activation of NF- $\kappa$ B. It induces the expression of adhesion molecules, chemokines and procoagulant molecules (4). This activated phenotype with upregulation of the proinflammatory genes is essential for attracting leukocytes, activating the immune system to counteract offending organism and limit the invasion. Even in the case of strong ECs stimulation, the ultimate goal of ECs is to survive which leads the cells to add to their constitutively expressed protective system by upregulation of a new set of protective genes, including those for A1 and A20 (9, 10). So, in the »super-protected« cells the inducible protective rejoinder becomes sufficient to function as a negative-feedback mechanism to turn off the proinflammatory response when no longer needed. This will restore the quiescent phenotype to the ECs. However, in extreme conditions such as uncontrolled septic shock or xenograft rejection, even the high levels of protection provided by the combination of the constitutively expressed and induced protective genes is not sufficient to counteract the intensity of the stimulation leading to the loss of

ECs. Therefore, the regulation of gene expression by using a potent and specific inhibitor (such as p65 RHD) of NF- $\kappa$ B – mediated induction of a number of genes, such as I kappa B- $\alpha$ , IL-8, E-selectin, P-selectin and tissue factor in ECs provides the basis for a novel therapeutic approach to the pathologic effects of ECs activation (8).

Endothelial cell response may unfold through three different types: stimulation of a rapid response of resting ECs initiated by agonists such as histamine; activation, a slower protein synthesis-dependent response initiated by inflammatory cytokines, and injury. The first two responses are normal functions of ECs. Injury can either produce endothelial necrosis or may lead to endothelial dysfunction and this cell sub-lethal injury may be produced by complement or cytolytic T-cells. As it has been already mentioned the most often the activation is mediated by cytokines. Cytokines are low-molecular-weight proteins, produced by different cells, which can be taken as a language of intercellular communication, that can act locally or at a distance, operate in a network, and be repressed or modified by various mechanisms. The ECs are both a target for cytokines and a source.

A large number of aetiologically distinct pathogens can be directly or indirectly involved in ECs activation: viruses (14–18), bacteria (19, 20), protozoa (21), rickettsiae (22), toxins (23, 24), but ECs can be a target for angiogenic signals in neoplasia (1) as well as a major target for immune reaction directed against alloantigens or xenoantigens (25, 26). Some of them infect ECs (like viruses), the others interact with ECs (viruses, gram-positive and gram-negative bacteria and their products, anti-EC antibodies) and induce or modify cytokine production (1) (Table I). By interactions between vascular endothelium, platelets and leukocytes or lymphocytes a signal exchanges, adhesion molecule expression and secretion of chemiotactic mediators occur initiating the immuno/inflammatory reaction. The cells intervening in the precocious infla-

mmatory phase are tissular mastocytes and platelet-liberating mediators (histamine) and neutrophil cells responsible for vascular injuries induced by oxygen free radicals. The monocytes, platelets and lymphocytes liberate cytokines early, which appears to be important in activation and production of an inflammatory response. Although there are indices suggesting that a cocktail of three cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ ) (27), is necessary for nitric oxide synthase (NOS) induction most data showed that ECs activation can be triggered by a mixture of two cytokines especially interleukin-1 and tumour necrosis factor (TNF) (1, 3). First, they induce the expression of a functional programme related to thrombosis and inflammation. Exactly, they stimulate procogulant activity (28), inhibit the thrombomodulin/protein C anticoagulation pathway and block fibrin dissolution via stimulation of the type I inhibitor of plasminogen activator. IL-1 and TNF also induce production of autocooids in ECs, including prostanoids, platelet-activating factor (PAF) and nitric oxide (NO). PAF expressed on ECs surface cooperates with adhesion molecules in leukocyte transmigration and can prime or activate circulating cells or ECs themselves. It is also a secondary mediator of angiogenesis. Prostanoid synthesis is dependent on the induction of phospholipase A<sub>2</sub> and cyclooxygenases. Furthermore, cytokines (TNF and IL-1) induce synthesis and secretion endothelial adhesion molecules such as ICAM-1, VCAM-1 (29) and E-selectin which mediate leukocyte recruitment to sites of inflammation (Table II). They also activate the fibroblasts and ECs that produce, among others, free radicals and other chemiotactic cytokines some of which (IL-8 and an additional degranulating factor not accounted for by IL-8) can induce neutrophil degranulation (30) and stimulate oxidative stress and formation of free radicals. IL-1 and TNF induce production of IL-6, colony-stimulating factors and IL-1 itself (31). *In vitro* culture of ECs results in spontaneous expression of IL-1 $\alpha$  and refraction of the response to exogenous IL-1 (32). Endothelial cells express both p55 and p75 TNF re-

Table I Human diseases in wich cytokine-EC interactions play a prominent role

Selected disease	Mediators	Response/function
<b>Infection</b>		
Virus (EBV)	IL-6	B cell growth
Rickettsiae	IL-1, IL-8, IL-6	Vasculitis
Bacteria:		
systemic (SIRS)	TNF, IL-1	Hypotension, thrombosis, organ failure
local (HUS)	Gb3, TNF, IL-1	Selective vascular damage (e.g. kidney)
Protozoa (malaria)	TNF/adhesion molecules	Cerebral malaria
<b>Cancer</b>		
Primary	Growth factors, TNF/IL-1, fibrin, chemokines	Angiogenesis (+/-)
Metastatic	TNF/IL-1, chemokines, adhesion molecules	Metastasis
<b>Atherosclerosis and cardiovascular disease</b>	MCP-1, inflammatory cytokines	Monocyte recruitment, amplification of tissue damage
<b>Autoimmunity</b>	AECAs, cytokines	Vasculitis

Table II Chemokines produced by endothelial cells

Family	Molecules	Endothelium	Principal inducers	Principal targets
CXC	IL-8	Diverse	LPS, IL-1, TNF, IL-6/IL-6R, IL-4, IL-13, IL-7, thrombin, hypoxia	Neutrophil
	ENA 78 GRO- $\alpha$ IP-10	HUVEC HUVEC Endothelium <i>in vivo</i>	Similar to IL-8 Similar to IL-8 LPS, IFN- $\gamma$	Neutrophil Neutrophil Monocyte, T
CC	MCP-1 MCP-3	Diverse HUVEC	Similar to IL-8, IFN- $\gamma$ Similar to IL-8	Monocyte, act T, act NK, Ba Monocyte, act T, act NK, DC, Ba, Eo
	RANTES Fractalkine	Diverse, <i>in vivo</i> HUVEC	TNF, IFN- $\gamma$ IL-1	Monocyte, act T, act NK, DC, Ba, Eo Monocyte

ceptor (TNFR), the latter being the most abundant on the cell membrane. The p55 is expressed at much lower levels on the membrane itself but is more abundant overall, mainly detectable in the Golgi and cytoplasmic vacuoles. TNF activates ECs predominantly via p55, while the contribution of p75 is best observed at low TNF concentration indicating that this molecule can present TNF at low concentration to p55 (a phenomenon termed »ligand passing«). The transmembrane form of TNF is the prime ligand of p75 and it may play an important role in interactions between ECs and monocytes. ECs express only the type I IL-1R and do not express the type II decoy receptor either under resting conditions or during activation (1).

Under the influence of inflammatory mediators some other enzymes are also activated, including nitric oxide synthase (NOS) which plays an important role in activated ECs via the production of nitric oxide (NO). Resting ECs constitutively express an endothelial-specific isoform of nitric oxide synthase (e-NOS) which continuously synthesizes NO from L-arginine (33). The most important stimuli are physical factors such as shear stress and pulsatile stretching of the vessel wall as well as circulating and locally released vasoactive substances. The endothelium can be taken as a biosensor reacting to a large variety of stimuli and therefore maintaining adequate NO release (34). Besides the essential role in the regulation of vascular wall tonus NO mediates a variety of biological functions such as neurotransmission, immunity and cytotoxicity. ECs can express both, Ca<sup>2+</sup> – dependent constitutive NOS (eNOS) and Ca<sup>2+</sup> – independent inducible NOS (iNOS) (35). In unstimulated ECs, eNOS is targeted to specific microdomains in the plasma membrane called caveolae, where eNOS is associated with a scaffold protein caveolin, resulting in tonic inhibition of the enzyme activity (36–40). The elevation of Ca<sup>2+</sup>

induced by Ca<sup>2+</sup> – elevating agonists stimulates the binding of calmodulin to eNOS challenging the dissociation of the enzyme from caveolin and thereby its activation (40). However, there is evidence that shear stress or ceramide can induce Ca<sup>2+</sup> – independent activation of eNOS (41, 42). In some situations the activity as well as the amount of constitutive eNOS is increased showing that this isoform can also be induced. Although it was thought that the iNOS may be induced only in pathological conditions there is also evidence that it may be induced in physiological settings such as pregnancy, treatment with oestradiol (43), shear stress and chronic exercise. In activated ECs iNOS can be induced producing much higher levels of NO than those present under physiological conditions, that is implicated in the pathogenesis of a wide variety of diseases involving endothelium. iNOS is highly regulated by cytokines, some of which promote and others inhibit the induction of the enzyme. Stimulatory cytokines increase iNOS mRNA (35). Nitric oxide synthase gene expression, mRNA stability, and protein synthesis or degradation are all amenable to modification by cytokines or other agents such as growth factors. For example, transforming growth factor- $\beta$  (TGF- $\beta$ ) reduces cytokine-induced iNOS activity by inhibiting iNOS mRNA translation and increasing iNOS protein degradation, while interleukin-4 interferes with iNOS transcription (44). Disruption of genes encoding IFN- $\gamma$ , part of its receptor or an IFN regulatory factor results in a phenotypic deficiency in iNOS expression (35).

Except of a variety of functions triggering by NO, ECs produced – NO also has an autocrine function. It inhibits cytokine-induced expression of adhesion molecules and cytokine production by ECs by inducing and stabilizing of NF- $\kappa$ B inhibitor (45, 46). Thus, constitutively produced NO may tonically inhibit the expression of NF- $\kappa$ B – dependent proinflammatory genes and attenuate the proinflammatory response of ECs.

In conclusion, there is a close inter-dependence between cytokines and NO/ NOS system; its understanding is important in human pathologies involving a stimulation or an activation of endothelium. Novel

strategies to preserve endothelium in its quiescent and functional state offer a new promising therapeutic approach in medical practice.

## MEĐUZAVISNOST CITOKINA I NO/NOS SISTEMA U »MIRUJUĆIM« I AKTIVISANIM ENDOTELNIM ČELIJAMA

Vidosava B. Đorđević<sup>1</sup>, Tatjana Cvetković<sup>1</sup>, Ivana Stojanović<sup>1</sup>, Vladan Čosić<sup>2</sup>,  
Slavica Kundalić<sup>2</sup>, Lilika Zvezdanović<sup>2</sup>, Tatjana Jevtović-Stoimenou<sup>1</sup>

<sup>1</sup>Biohemijski institut, Medicinski fakultet, Niš

<sup>2</sup>Centar za medicinsku biohemiju, Klinički centar, Niš, Srbija i Crna Gora

*Kratak sadržaj:* Citokini su heterogena i multifunkcionalna grupa molekula koji se sintetišu u različitim humanim ćelijama. Po strukturi su peptidi (često glikozilisani) koji omogućavaju interćelijsku komunikaciju i kontrolu unutrašnje sredine ćelija u kojima deluju. Produkuju ih ćelije koje učestvuju u imunom odgovoru, inflamaciji, hematopoezi, reparaciji tkivnog oštećenja i sistemskom odgovoru na oštećenje. Imunitet, inflamatorne reakcije i hemostaza se karakterišu uskom interakcijom imuno-kompetentnih ćelija i vaskularnog endotela. Vaskularne ćelije su ciljne ćelije za citokine ali ih mogu i proizvoditi. Spektar odgovora endotelne ćelije na citokine je širok i raznolik, jer različiti citokini aktiviraju različite, ali mogu i iste, grupe funkcija. Pod normalnim (resting) uslovima endotelne ćelije konstitutivno ekspimiraju izvesne protektivne gene čiji je cilj da održe endotelne ćelije u stanju mirovanja sprečavanjem aktivacije NF-κB i ispoljavanjem anti-apoptičnog delovanja. U takvom stanju endotelne ćelije mogu da ispolje barijernu i antikoagulantnu funkciju čak i u prisustvu malih količina stimulanasa. Kada se endotelne ćelije izlože brojnim stimulansima kao što su TNF, IL-1, endotoksin ili ksenoreaktivna antitela i komplemen, koji se sreću kod infekcija, odbacivanja grafta ili autoimunih bolesti kao što je vaskulitis, NF-κB indukuje ekspresiju adhezionih molekula uključujući E-selektin, hemokine kao što je IL-8 i prokoagulantne molekule kao što je TF. Pored indukcije ekspresije funkcionalnog programa koji se odnosi na trombozu i inflamaciju IL-1 i TNF takođe indukuju produkciju lokalnih medijatora uključujući i azot-monoksid (NO). U endotelnim ćelijama su prisutne dve izoforme azot-monoksid sintaze, inducibilna (iNOS) tip II i konstitutivna (tip III), koje katalizuju konverziju arginina u citrulin i NO. Stvaranje NO je ubikvitami biohemijski put koji učestvuje u regulaciji neurotransmisije, vazodilatacije, imuniteta i citotoksičnosti. U toku inflamatorne reakcije NO koji se proizvodi u endotelnim ćelijama ispoljava lokalna dejstva preko inhibicije citokinima-indikovane ekspresije adhezionih molekula i citokina u endotelnim ćelijama. Takođe, u inflamaciji ispoljava protektivno dejstvo i preko inaktivacije NADPH oksidaze i posledične produkcije superoksida. S druge strane, ima dokaza da NO doprinosi tkivnom oštećenju u inflamaciji i imunim bolestima budući da je ključna komponenta citostatske/citotoksične funkcije imunog sistema. Oštećenje ciljnih ćelija pomoću NO oslobođenog iz aktivisanih makrofaga ili endotelne ćelije može ići po tipu nekroze ili programirane ćelijske smrti.

*Ključne reči:* endotelne ćelije (ECs), citokini, azot monoksid (NO), azot-monoksid sintaza (NOS)

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