NF-κB and ERK cooperate to stimulate DNA synthesis by inducing ornithine decarboxylase and nitric oxide synthase in cardiomyocytes treated with TNF and LPS

Benedetta Tantini[a,*], Carla Pignatti[a], Monia Fattori[a], Flavio Flamigni[a], Claudio Stefanelli[a], Emanuele Giordano[a], Marta Menegazzi[b], Carlo Clô[c], Claudio Marcello Caldarera[a]

[a] *Department of Biochemistry 'G. Moruzzi', School of Medicine, University of Bologna, via Irnerio 48, 40126 Bologna, Italy*
[b] *Institute of Biological Chemistry, University of Verona, 37134 Verona, Italy*
[c] *Institute of Biological Chemistry, School of Medicine, University of Parma, 43100 Parma, Italy*

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Abstract We previously reported that tumor necrosis factor-α (TNF) and lipopolysaccharide (LPS) stimulate DNA synthesis in chick embryo cardiomyocytes (CM) via nitric oxide and polyamine biosynthesis. Here we show an involvement of nuclear factor-κB (NF-κB) in the induction of nitric oxide synthase (NOS) and ornithine decarboxylase (ODC), the key enzyme in polyamine biosynthesis. In addition NF-κB activation appears to favor survival of CM by reducing caspase activation. TNF and LPS also stimulate phosphorylation of extracellular signal-regulated kinase (ERK), which is required for the changes in ODC and caspase activity, but not for NOS induction or NF-κB activation. In conclusion, these results indicate that NF-κB, in cooperation with ERK, plays a pivotal role in the growth stimulating effects of TNF and LPS, leading to the induction of both ODC and NOS and to the reduction of caspase activity.

Key words: Tumor necrosis factor-α; Lipopolysaccharide; Nuclear factor-κB; Extracellular signal-regulated kinase; DNA synthesis; Cardiomyocyte

1. Introduction

Tumor necrosis factor-α (TNF) and lipopolysaccharide (LPS) share a common spectrum of cellular activities including growth, differentiation and apoptosis [1,2] and can evoke inflammation and cardiovascular responses [3,4]. The molecular mechanisms responsible for the multiple biological activities of TNF and LPS are not completely clarified, but these are probably due to their ability to activate multiple signal transduction pathways [5,6], including those involving extracellular signal-regulated kinase (ERK) and other mitogen-activated protein kinases (MAPKs), which play critical roles in cell proliferation and survival [7]. TNF and LPS also activate nuclear factor-κB (NF-κB), a factor that promotes transcription of a large family of genes [8]. NF-κB is a member of Rel family of proteins and is typically an heterodimer composed of a p50 and p65 (Rel A). In quiescent cells NF-κB resides in the cytosol in a latent form bound to an inhibitor protein (I-κB). Stimulation of the cell with various cytokines, LPS or oxidants, triggers a series of signalling events that ultimately leads to the phosphorylation and the proteolytic degradation of I-κB, and activation of NF-κB. The phosphorylation of I-κB is elicited by an I-κB kinase, which can be activated by MAPKs [9,10], while the proteolysis of I-κB is mediated by the ubiquitin-proteasome pathway of protein degradation. The degradation of I-κB triggers the translocation of NF-κB from the cytoplasm to the nucleus where it regulates the expression of multiple genes. It has been reported that TNF activation of NF-κB protects against apoptosis [11] and stimulates the proliferation of some cell types [12,13], whereas TNF-induced apoptosis involves the recruitment of the signal transducer FADD to type I TNF receptor [14]. A few NF-κB target genes that regulate apoptosis have been identified [15], however little is known about the targets of NF-κB relevant for its proliferative effects.

Cardiomyocyte (CM) proliferation occurs during embryonic and neonatal development and, according to recent evidence [16], even in the adult heart, particularly under certain pathological conditions such as myocardial infarction. Therefore it may be important to identify extracellular mediators and intracellular pathways leading to DNA replication in CM. Our previous report [17] indicates that in chick embryo CM cultures, TNF and LPS exert a mitogenic effect through a pathway involving polyamine and nitric oxide (NO) biosyntheses. In the present study we report data supporting the involvement of NF-κB in the induction of ornithine decarboxylase (ODC), the key enzyme in polyamine biosynthesis [18], and nitric oxide synthase (NOS). Simultaneously, NF-κB activation appears to favor survival of CM by reducing caspase activation, a critical event in the executive phase of apoptosis. Activation of ERK may also be involved in these events.

2. Materials and methods

2.1. CM cultures

Preparation of monolayer cultures of spontaneously beating em-

*Corresponding author. Fax: (39)-51-2091224.
E-mail address: tantini@biocfarm.unibo.it (B. Tantini).

Abbreviations: CM, cardiomyocyte; DFMO, α-difluoromethylornithine; ERK, extracellular signal-regulated kinase; I-κB, inhibitor protein; LLL, *n*-acetylleucilleucinal; L-NMMA, N^G-monomethylarginine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NOS, nitric oxide synthase; NF-κB, nuclear factor-κB; ODC, ornithine decarboxylase; PDTC, pyrrolidine dithiocarbamate; STS, staurosporine; TNF, tumor necrosis factor-α

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bryo CM from the hearts of 10 day-old chick embryos was carried out as previously described [17]. This method resulted in preparations containing >95% CM, as assayed by immunofluorescence staining with antibodies against cardiac myosin heavy chain, which began to beat spontaneously after 2 days in culture. Confluent cultures were then serum-starved for 20 h before treatment with the different drugs, as described in the legends. Cell death was evaluated by trypan blue exclusion, with the percentage of cell death being defined as the percentage of cells that include the dye.

2.2. Enzymatic assays

The ODC and NOS activities were measured by estimation of the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine or monitoring L-[^{3}H]citrulline formation from L-[2,3-^{3}H]arginine as previously described [19]. The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-DEVD-AMC during a 15-min incubation at 37°C, as detailed elsewhere [20]. Since the DEVD sequence represents a substrate for caspase-3 and other members of the caspase family, this activity will be referred to as caspase activity. Data are expressed as pmol/mg protein/min.

2.3. [^{3}H]Thymidine incorporation

DNA synthesis was quantified by [^{3}H]thymidine incorporation of subconfluent CM cultures. The cells, maintained for 20 h in serum-free Dulbecco's modified Eagle's medium, were then treated with the different drugs, and pulsed during the last 2 h with [^{3}H]thymidine [17]. Data are expressed as % of the radioactivity measured under basal conditions.

2.4. Western blot analysis and electrophoretic mobility shift assay (EMSA)

Western blot analysis of phosphorylated ERK was performed by using phosphospecific antibody as described elsewhere [21]. DNA binding activity of NF-κB was assayed in nuclear extracts as previously described [22].

2.5. Statistical analyses

Values are given as means ± S.D. All experiments were performed with at least three independent CM cultures.

3. Results and discussion

3.1. NF-κB mediates induction of ODC and NOS and reduction of caspase activity in TNF and LPS treated CMs

We have first ascertained whether NF-κB may be activated in TNF and LPS treated CM. DNA binding activity of the transcription factor NF-κB was evaluated by EMSA. Fig. 1A shows that serum-starved CM has a low basal level of NF-κB activity, which increases after 30 and 60 min of TNF and LPS treatment. When the cells were pre-incubated with *n*-acetylleucilleucinal (LLL), an inhibitor of the ubiquitine-26S proteasome degrading I-κB [23], a complete prevention of TNF and LPS mediated NF-κB activation was observed. The gel shift bands were specific NF-κB–DNA–protein complexes, because the addition of excess unlabelled NF-κB oligonucleotide to nuclear extract specifically abolished the NF-κB signal.

We have previously shown that TNF and LPS exert a mitogenic effect in chick embryo CM through a pathway that involves an increase in polyamine and NO biosyntheses [17]. In fact ODC and NOS activities were strongly induced after treatment of CM with TNF and LPS, as also reported in Fig. 1B. The increase of NOS activity may result from induction of iNOS, as shown in other experimental systems [24]. It has been described that the promoter region of the iNOS gene contains the consensus sequence for the binding of NF-κB, which is known to control iNOS expression in a variety of cell types [25]. Sequences in the ODC gene that show similarities to binding sites for NF-κB, have been noted [26], however we are not aware of published reports showing the actual involvement of this transcription factor in the regulation of ODC expression.

In order to determine whether TNF- and LPS-induced ODC and NOS activities in CM were mediated by NF-κB activation, experiments were performed with the aid of three structurally different inhibitors of NF-κB, which act by different mechanisms: LLL, already mentioned above, pyrrolidine

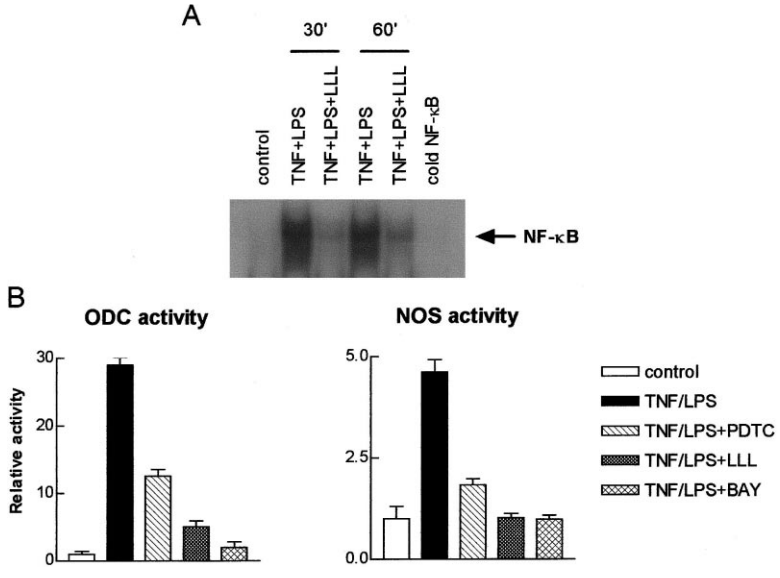

Fig. 1. NF-κB is activated in confluent chick embryo CM treated with TNF and LPS and is required for ODC and NOS induction. A: Serum-starved CM were treated with 500 U/ml TNF and 10 μg/ml LPS for 30 and 60 min. 5 μM LLL was added 2 h before TNF and LPS. Cell extracts were analyzed for DNA binding activity of NF-κB by EMSA. B: Serum-starved CM were treated with 500 U/ml TNF and 10 μg/ml LPS for 4 h (ODC) or for 8 h (NOS). 10 μM PDTC, 5 μM LLL or 10 μM Bay 11-7082 were added 2 h before TNF and LPS. The results are expressed as relative activity with respect to the activities of control cells, taken as 1.0. Control ODC activity was 0.30 ± 0.1 pmol/mg protein/min. Control NOS activity was 2.4 ± 0.3 pmol/mg protein/min.

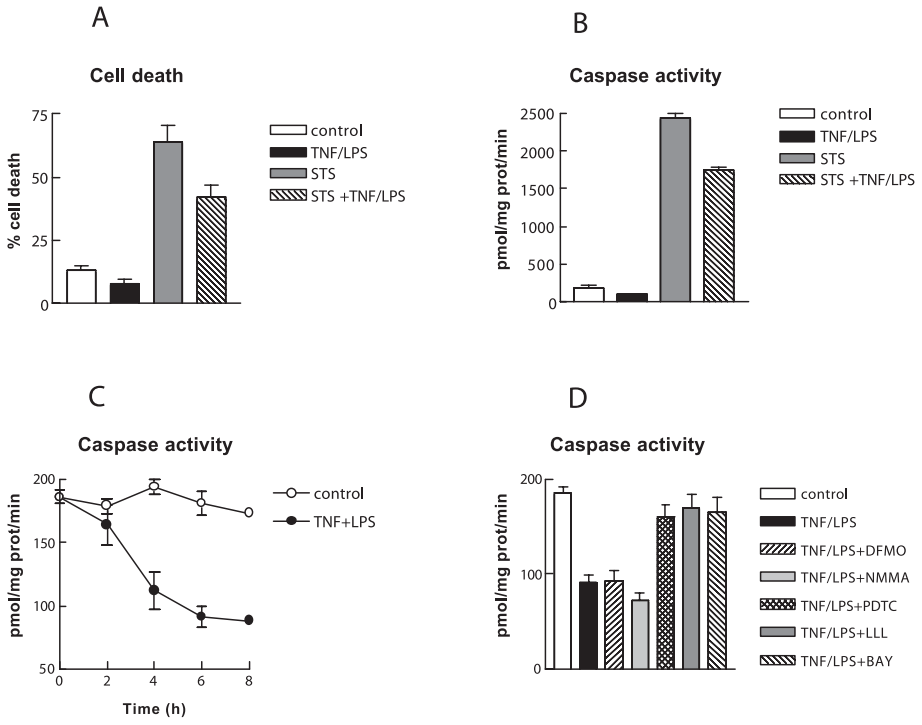

Fig. 2. TNF and LPS reduce cell death and caspase activity in confluent chick embryo CM. A, B: Serum-starved CM were treated for 24 h (A) or for 6 h (B) with 500 U/ml TNF and 10 μg/ml LPS in the presence or absence of 1 μM STS. C: Serum-starved CM were treated with 500 U/ml TNF and 10 μg/ml LPS. D: Serum-starved CM, pre-treated for 20 h with 4 mM DFMO, for 1 h with 100 μM L-NMMA or for 2 h with 10 μM PDTC, 5 μM LLL or 10 μM Bay 11-7082 were then incubated with 500 U/ml TNF and 10 μg/ml LPS for 6 h.

dithiocarbamate (PDTC), which blocks NF-κB activity by an antioxidant mechanism [27] and Bay 11-7082, a specific IκBa-kinase inhibitor [28]. Fig. 1B indicates that pre-incubation of the cells with each of the different inhibitors prevents the induction of ODC and NOS activities by TNF and LPS, suggesting that NF-κB activation is involved in the pathway by which TNF and LPS lead to ODC and NOS induction.

On the other hand substantial evidence indicates that activation of cell proliferation sensitizes cells to apoptosis, which may proceed following cell damage or even spontaneously, unless the mitogenic stimuli provide survival signals [29]. It

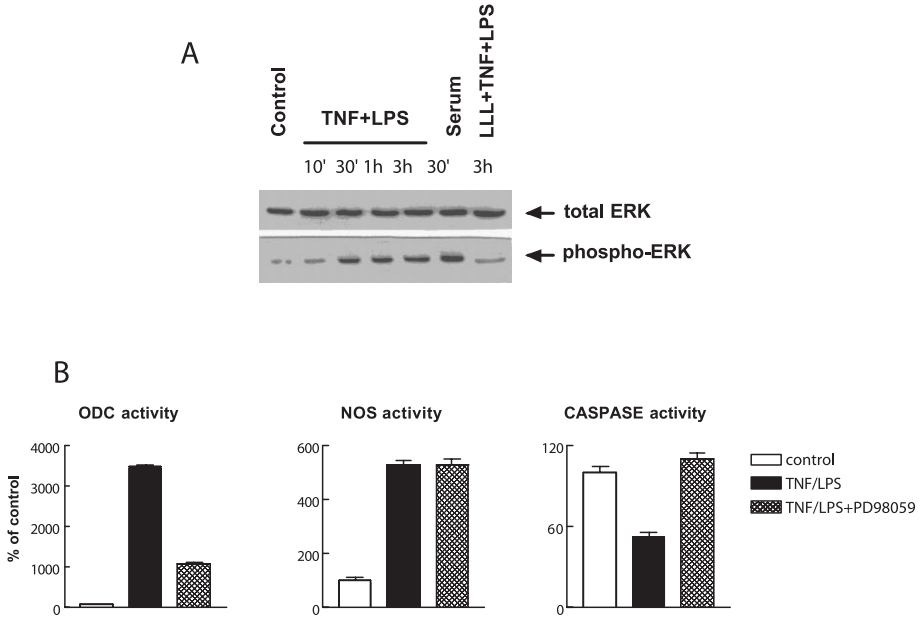

Fig. 3. Activation of ERK in confluent chick embryo CM treated with TNF and LPS and effect of PD98059 on ODC and NOS induction and caspase changes. A: Serum-starved CM were treated with 500 U/ml TNF and 10 μg/ml LPS or 10% serum. 5 μM LLL was added 2 h before TNF and LPS. Cell extracts were analyzed by Western blot by using specific antibodies. B: Serum-starved CM were treated for 4 h (ODC), 8 h (NOS) or 6 h (caspase) with 500 U/ml TNF and 10 μg/ml LPS. 50 μM PD98059 was added 1 h before TNF and LPS.

is also known that TNF can favor either cell death or survival in various cell types, including CM, depending on the developmental stage, association with other stimuli and other experimental conditions [1,3,6]. Therefore we have investigated whether, in our model of chick embryo CM, TNF and LPS affect cell viability. Fig. 2A reports how the treatment with TNF and LPS for 24 h caused a reduction of the fraction of dead cells, both under basal conditions (by 43%) and after addition of staurosporine (STS), a widely used and potent inducer of apoptosis. Furthermore, the pro-survival effect of TNF and LPS was accompanied by corresponding changes in the activity of caspases, proteolytic enzymes that are known to play a central role in apoptosis. In fact Fig. 2B shows that TNF and LPS treatment reduced both basal and STS-induced caspase activity. The time-course of caspase activity in chick embryo CM treated only with TNF and LPS is depicted in Fig. 2C, showing a progressive reduction up to 8 h of incubation (Fig. 2C). This effect was completely prevented when the cells were pre-incubated with the NF-κB inhibitors LLL, PDTC or Bay 11-7082 (Fig. 2D), indicating that even caspase inhibition by TNF and LPS is an NF-κB mediated event. It has been reported that in some cell systems NO and polyamines may play protective roles in apoptosis [30,31]. However, Fig. 2D shows that the inhibition of caspase activity by TNF and LPS is a polyamine and NO independent phenomenon. In fact the pre-treatment of CM cultures with α-difluoromethylornithine (DFMO), a specific ODC inhibitor, or with N^G-monomethylarginine (L-NMMA), a competitive inhibitor of NOS, was without effect on the inhibition of caspase activity by TNF and LPS.

3.2. ERK activation is required for the induction of ODC and the reduction of caspase activity

Another important signaling pathway that may be activated by TNF and/or LPS, is that involving ERK, which is considered to play an essential role in mitogenesis and, in some cell types, even in cell survival [7]. Therefore, the activation/phosphorylation of ERK was investigated in our experimental system. By using specific antibodies against total or phosphorylated ERK, a single immunoreactive band was detected (Fig. 3A). The total content of ERK did not vary following 3 h of TNF and LPS treatment; however, the active, phosphorylated ERK, very low in quiescent CM, increased markedly within 30 min of TNF and LPS treatment, although not as much as after serum stimulation, and remained high for up to 3 h.

Given the importance of ODC and NOS for the mitogenic action of TNF and LPS treatment, we investigated the effect of PD98059, a specific MEK inhibitor widely used to dissect the ERK pathway [32], on the induction of ODC and NOS (Fig. 3B). PD98059, which prevented TNF- and LPS-induced ERK activation (not shown), proved to be a potent inhibitor of ODC induction, but did not influence NOS induction. We have previously shown that ERK activation is required for ODC expression and induction in serum-stimulated leukemia cells [21] and in ECV304 cells stimulated with some ligands of G-protein coupled receptors [33]. In addition PD98059 reversed the TNF and LPS effect on caspase activity in CM (Fig. 3B), supporting a pro-survival effect of ERK. In some cell systems the anti-apoptotic action of ERK has been related to its ability to affect phosphorylation and expression of Bcl-2 family members [7], which in turn may control cytochrome *c* release and thus caspase activation.

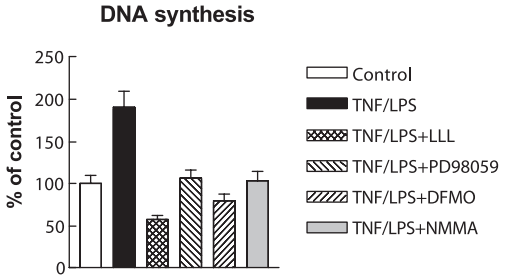

Fig. 4. TNF and LPS increase DNA synthesis in confluent chick embryo CM. Effect of PD98059, LLL, DFMO or L-NMMA. Serum-starved CM were incubated for 20 h with 500 U/ml TNF and 10 μg/ml LPS in the absence or presence of 50 μM PD98059, 5 μM LLL, 4 mM DFMO or 100 μM L-NMMA.

3.3. NF-κB and ERK cooperate to stimulate DNA synthesis

Fig. 4 shows that the treatment of CM with PD98059, LLL, DFMO or L-NMMA, inhibited the stimulation of DNA synthesis elicited by TNF and LPS. This indicates that activation of ERK and NF-κB, as well as induction of ODC and NOS, are all events required for the stimulation of DNA synthesis by TNF and LPS. Since blockade of either ODC or NOS pathway is sufficient to prevent the increase in DNA synthesis, it is not surprising that PD98059 and LLL, which also inhibit ODC and/or NOS induction, reproduce the same result.

It has been reported that in some experimental models ERK stimulation leads to NF-κB activation [9,10]. However treatment of CM with PD98059 was not able to prevent NF-κB activation by TNF and LPS, as judged by gel shift assay (not shown), indicating that under our experimental conditions NF-κB is not placed downstream of ERK. This would be in agreement with the finding that NF-κB blockade prevents the TNF- and LPS-induced changes in ODC, NOS and caspase activities, whereas inhibition of the ERK pathway prevents only the ODC and caspase modifications. On the other hand the increase of ERK phosphorylation was almost completely abolished in the presence of LLL (Fig. 3B), whereas it was not by Bay 11-7082 (not shown). These latter data suggest that the proteasome, but not NF-κB may be involved in the control of ERK phosphorylation.

In conclusion the present results are consistent with a picture where NF-κB plays a pivotal role in the mitogenic effect of TNF and LPS, leading to the induction of both ODC and NOS enzymes. We have previously shown [17] that in turn a rise in cGMP content mediates the effects of polyamines and NO accumulation. In addition TNF and LPS provoke ERK stimulation that is also required for ODC induction and DNA synthesis. Simultaneously, NF-κB and ERK cooperate even to reduce caspase activity, thus favoring cell survival. Finally, according to the experiments with PD98059 and Bay 11-7082, NF-κB is not located downstream of ERK in this system, but NF-κB and ERK may be placed on separate pathways that converge to favor proliferation and survival of CM.

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