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## The NEP protein of influenza A virus activates the raf/MEK/ERK pathways to regulate the TNF- $\alpha$ gene expression

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The severity of the influenza virus disease has been associated with the expression of the tumor necrosis factor alpha (TNF- $\alpha$ ); however, it is not yet clear as regulates the expression of TNF- $\alpha$  during infections by the influenza virus. TNF- $\alpha$  plays relevant roles both inducing and regulating immune response to virus infections and is considered an anti-influenza cytokine; nonetheless, highly pathogenicavian influenza viruses replicate in the presence of high concentrations of TNF. To analyze the regulation of TNF- $\alpha$  gene expression in the infection by influenza A virus, in this work it was studied the influence of NEP and NS1 proteins on their expression. Both of them are encoded in genomic segment 8. NS1 protein is involved in the evasion of innate immunity. NEP (protein of export nuclear) participates in the regulation of both virus transcription and replication via interaction with viral polymerase complex. In this study, the NS1 and NEP ORF of influenza A virus were cloned to produce pCAGGS-NS1 (NS1PR8) and pCAGGSNEPpdm (NEPpdm) vectors. NS1 and NEP was overexpressed in HeLa cells. Two promoter regions of TNF- $\alpha$  gene were cloned in a luciferase reporter vector, pGL4-fullTNF and pGL4proxTNF of 1200 pb and 200 pb, respectively. The bioinformatics analysis of these regions found response elements to NF- $\kappa$ B and raf/MEK/ERK pathways; on pGL4proxTNF vector were studied the binding sites of transcription factors NFAT/Ets, Egr, SP1 and CRE. HeLa cells were co-transfected with pGL4-pTNF reporter vectors, NS1PR8 and NEPpdm, and the transfection control (pGL4.72 hRlucCP). After 5 h, cells were treated with different compounds, which specifically inhibit Raf/MEK/ERK (U0126), NF- $\kappa$ B (Bay-11-7082) and PI3K (Ly294-002) signaling pathways. NS1PR8 showed reduced TNF- $\alpha$  promoter activity compared to control mean ( $26.4 \pm 1.2$ ,  $20.4 \pm 0.4$  and  $27.1 \pm 3.2$ ). NEPpdm induced notable activation of the full-length v promoter ( $77 \pm 1.5$ ,  $87.7 \pm 0.4$  and  $83.5 \pm 2.9$ ;  $p < 0.001$ ) compared with control cells at 12, 18 and 24 h post-transfection. The activity of proximal TNF- $\alpha$  promoter was 15-fold lower compared with the activity of the full length promoter but the activity was 1-, 1.8-, and 2-fold higher compared to control cells at 12, 18 and 24 h post-transfection, respectively. Blocking of Raf/MEK/ERK pathway reduced the activity of TNF- $\alpha$  promoter ( $41.5 \pm 3.2$  and  $80.6 \pm 7.4$ , respectively) compared to mock-treated control (70%); and blocking of NF- $\kappa$ B reduced 35% the activity. The data obtained in this study shows that the NEP protein is involved on the regulation of the TNF- $\alpha$  gene expression in the infection with the influenza A virus, by the activation of the MAPK and probably the NF- $\kappa$ B signal pathways.

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