


# Time course study of ERK1/2 activity and cell viability in lipopolysaccharide challenged PC12 cells



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## ABSTRACT

**Introduction:** Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are important members of mitogen-activated protein kinases (MAPK), which are actively involved in the shaping of cellular responses to different stimuli; however, these responses are somehow contradicting. It is believed that time is a crucial factor in the determination of these effects. Therefore, the present work was designed to obtain a more vivid view about the effect of time on ERK activity and its relation to cell viability.

**Methods:** In the first step, we challenged cultured PC12 cells with different doses of lipopolysaccharides (LPS) for different time intervals (3, 6, 24 and 48h) and the cell viability was checked by MTT test. Thereafter, we cultured the cells in 6-well plates and treated them with the effective dose (10µg/ml) for the abovementioned intervals and the level of ERK phosphorylation, as the active form, was assessed in the Western blotting analysis.

**Results:** The results showed that treating cells with 10µg/ml LPS reduces cell viability after 48h. While being ineffective in shorter periods of time, ERK activity has a fluctuating trend, so that it reaches the highest level at 6h, thereafter it declines to the lowest level at 24h and partially increases again at 48h.

**Conclusion:** These results imply that time is a determinant factor in the activity of ERK and single-point assessments may result in misinterpretation.

## Keywords:

Inflammation

Time factors

ERK1/ 2 MAPK

Lipopolysaccharides

PC12 cells

## Introduction

Extracellular signal-regulated kinase 1 (44 kDa) and 2 (42 kDa) (ERK1/2) or briefly ERK are members of the mammalian family of mitogen-activated protein kinases (MAPKs). The activated form of ERKs is the phosphorylated form on tyrosine and threonine residues. Phosphorylation of ERKs can be triggered in response to different stimuli such as cytokines, chemokines, growth

factors and oxidative stress (Cheng et al., 2013). Once phosphorylated, ERKs are implicated in the regulation of cellular processes such as differentiation and proliferation, cell survival and/or apoptosis (Abbasnejad et al., 2019; Cheng et al., 2013). In order to accomplish these diverse effects, ERK isoforms participate in shaping the response of different signaling pathways, such as regulation of inflammatory responses (Sabio and Davis,

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2014). In this regard, the question, which may arise is how a signaling molecule could contribute to such diverse and even opposing effects. It seems that time is an important factor in revealing these effects (Abbasnejad et al., 2019; Stanciu et al., 2000). Accordingly, it has been demonstrated that ERK can be a survival factor for the cell when activated in short periods, but its activation in longer periods can lead to cellular death (Li et al., 2002). Considering these points and in order to expand our understanding about the role that time play in forming cellular responses, we aimed to assess time-dependency of ERK activity and its correlation with cell viability in 4 different time points (3, 6, 24 and 48h) after challenged with LPS.

## Material and methods

### Chemicals

Hanks' Balanced Salt (HBS, H2387) and LPS (L2880) were purchased from Sigma–Aldrich. Cell culture material including 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), complete Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin (15140-122) were from Gibco. Antibodies against phospho-ERK antibody (Rabbit mAb #4377), Total ERK antibody (Rabbit mAb #4695), beta-actin antibody (Rabbit mAb #4970) and secondary HRP-conjugated antibody (Anti-rabbit IgG, HRP-linked Antibody #7074) were obtained from Cell Signaling Technology. Amersham ECL select™ (RPN2235) reagent kit was purchased from GE health care. Bovine serum albumin (BSA, 1120180100) and PVDF membrane (IPVH00010) were purchased from Millipore.

### PC12 culture

PC12 cells were obtained from the Neuroscience Research Center, Shahid Beheshti University of Medical Sciences. The cells were cultured in complete DMEM medium (with 10% FBS, 1% antibiotic mixture comprising penicillin-streptomycin) in T25 flasks which were preserved at 37°C in a 5% CO<sub>2</sub> incubator at 37°C and 95% humidified atmosphere. The medium of the cells was exchanged every 2 days. For the experiments, equal numbers of PC12 cells were seeded in 96 or 6 well plates. The ethics committee of Shahid Beheshti University of Medical Sciences approved the experimental protocol (IR.SBMU.MSP.REC.1395.280).

### Cell viability assay

In order to reach the toxic dose of LPS, cells were seeded in 96 well plates in DMEM containing 10% FBS. One day later, the culture medium was replaced with 1% FBS and cells treated with various dosages of LPS (500ng/ml, 1, 5 and 10µg/ml) in the time courses of 3, 6, 24 and 48h and cell viability was quantified by the colorimetric MTT assay. LPS (as a specific inducer for inflammation) was dissolved primarily in saline as a stock solution and then diluted in culture media to make final concentrations. At the end of treatment time, the cell culture medium was removed and changed with a DMEM media containing 0.5mg/ml of MTT. Then, the cells were returned into the incubator (37°C) for 4h, in this period, metabolically viable cells transform MTT into an insoluble formazan. Finally, the media was removed and the purple formazan crystals were dissolved in 100µl of DMSO and the absorbance of samples was measured at a wavelength of 570nm with a microplate reader.

### Western blot assay

Cells were lysed and homogenized in an ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors. After being incubated on ice for 30min, the lysates were centrifuged at 13,000 rpm for 30min at 4°C. Then whole protein concentrations of cells were measured by the Bradford method. In the next step, equal amounts of proteins were separated on 12% SDS-PAGE gel and transferred to PVDF membrane. Thereafter, the blots were blocked in 5% BSA for 1h at room temperature and then incubated with primary antibodies (P-ERK, T-ERK and beta-actin) overnight at 4°C. The membranes then were rinsed and probed with secondary antibody for 1.5h at room temperature. In the next step, in order to reveal the immunoreactive bands, ECL was added to the blots. Finally, the quantitative value of bands were obtained by Image J software.

### Statistical analysis

All data were expressed as mean±SEM. Results were analyzed by unpaired t-test and one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons.  $P<0.05$  was defined statistically significant. GraphPad Prism 6 Demo (GraphPad Software) program was used to data analysis.

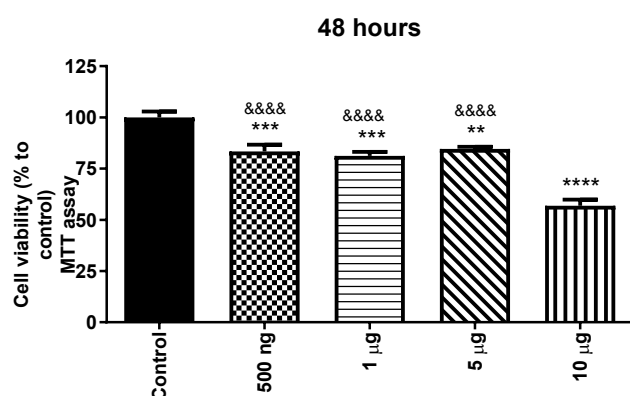
## Results

In order to reach the effective dose of LPS, we determined the cell viability of PC12 cells by MTT assay. To accomplish this, cells were incubated with LPS (four different doses: 500ng/ml, 1, 5 and 10µg/ml) for 48h. As depicted in Figure 1, analysis of cell viability after 48h implied that incubating of the cells with LPS in all doses resulted in decreased cell viability in comparison to control ( $F(4,33)=33.87$ ,  $P<0.0001$ ). However, cell death is more severe in 10µg/ml treated cells ( $P<0.0001$ ). More-

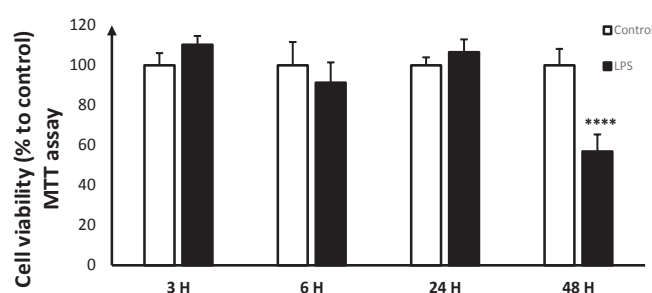
over, cells receiving 10µg/ml LPS had significantly lower cell viability compared to 500ng/ml, 1 and 5µg/ml LPS treated cells ( $P<0.0001$ ). Based on these results, we chose 10µg/ml and analyzed its effect on cell viability in 4-time points (3, 6, 24 and 48h). It is evident in Figure 2 that, there were no significant changes between the control and LPS groups unless the 48h group.

### The time-course study of ERK activity

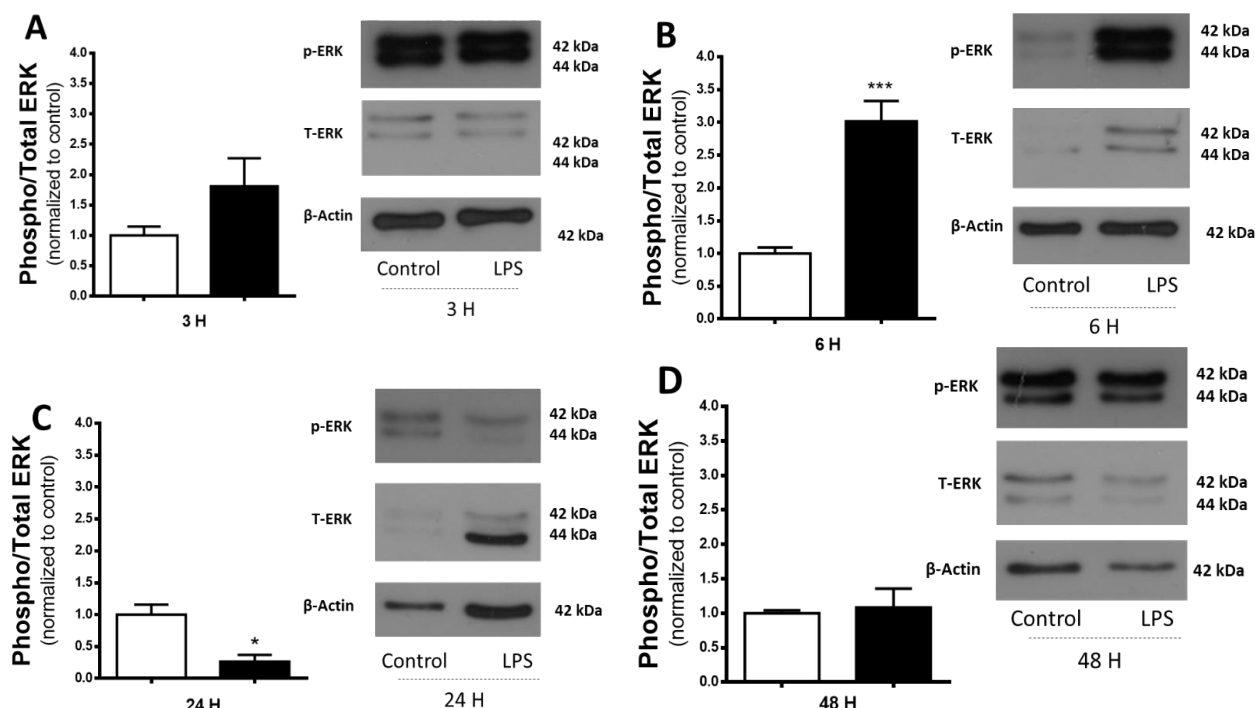
To evaluate the activity of ERK, phosphorylated and



**FIGURE 1.** Dose dependent study of LPS on cell viability in PC12 cells after 48h. Data are represented as mean±SEM. \*\* $P<0.01$ , \*\*\* $P<0.001$  and \*\*\*\* $P<0.0001$ , represent the difference between control and other groups. \*\*\*\* $P<0.0001$ , represents the difference between 10µg/ml and other groups.



**FIGURE 2.** Time dependent study of 10µg/ml LPS on cell viability in PC12 cells. Data are represented as mean±SEM. \*\*\*\* $P<0.0001$ , represent the difference between control and other groups.



**FIGURE 3.** Western blot analysis showing the time course effects of LPS on phosphorylated level of ERK; A, B, C and D are showing the comparative level of ERK phosphorylation 3, 6, 24 and 48h after changing media and LPS treatment. Data are represented as mean±SEM. \* $P<0.05$  and \*\*\* $P<0.001$ , represent the difference between control and LPS. Representative blot for each time is presented at right panel of each graph.

total forms of this kinase were assessed by Western blotting. The bands of phospho-ERK (p-ERK) and total-ERK (T-ERK) were detected at 42 and 44kDa. As showed in Figure 3, t-test analysis failed to significant difference between control and LPS after 3h ( $t=1.672$ ,  $df=4$ ,  $P=0.1699$ ). However, the result of analysis after 6h showed a significant increase in ERK activity in LPS treating group ( $t=6.248$   $df=6$ ,  $P=0.0008$ ). Interestingly, a significant decrease in ERK activity in the LPS group after 24h was detected ( $t=3.864$   $df=4$ ,  $P=0.0181$ ). Thereafter, ERK activity increased in the 48h group and became insignificant with the control group ( $t=.3007$   $df=6$ ,  $P=0.7738$ ).

## Discussion

In this report, we aimed to investigate the correlation between duration of inflammation, cell viability and the activity of ERK. The upper range of this time course study was 48h, this time was the first time point in which the LPS treatment caused significant cell death when compared to the control group. Our results showed that ERK phosphorylation, as an indicator for its activity, fluctuate in a time dependent manner. Several studies have revealed the involvement of ERK in inflammation. ERK induces inflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-8 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Kim, 2014; Kurosawa et al., 2000) and participate in various inflammatory disorders such as inflammatory osteolysis (Seo et al., 2010), cystic fibrosis (Verhaeghe et al., 2007), lung inflammatory diseases (Schuh and Pahl, 2009; Wuyts et al., 2003; Zhou et al., 2018) and arthritis (Thalhamer et al., 2008). In addition, it has been reported that inhibition of ERK phosphorylation can decrease neutrophil-dependent inflammation (Senger et al., 2017; Thalhamer et al., 2008). In contrast, Yong et al. have demonstrated that ERK inhibits IkappaB kinase (IKK) activity in endothelial cells, proving anti-inflammatory effects for this molecule (Maeng et al., 2006).

In the nervous system, ERK expression was reported in neurons, microglia, astrocytes and oligodendrocytes. Recently, it has been shown that ERK can control the release of many microglial proinflammatory mediators such as IL-1 $\beta$ , IL-6, COX-2, iNOS and TNF- $\alpha$  (Jeong et al., 2019; Deng et al., 2012; Kim et al., 2014; Weinstein et al., 2009). Furthermore, a recent study demonstrated that microglial ERK is hyperphosphorylated in Alzheimer's disease. Accordingly, it was

suggested that ERK plays a pivotal regulatory role in pro-inflammatory immune responses seen in this disease (Chen et al., 2019). As it was mentioned, ERK is also expressed in astrocytes and oligodendrocytes and plays critical roles in the inflammatory responses (Fields et al., 2013; Park et al., 2009; Parthasarathy and Philipp, 2014). As it was reviewed by Cruz and Cruz (2007), ERK pathway is also activated at the different neuronal levels, such as dorsal root ganglia, spinal cord and supraspinal centers and contributes to painful conditions that it could be a symptom of chronic inflammation. Consistently, ERK signaling in nociceptive spinal neurons might participate in inflammation mediated pain hypersensitivity (Zhang et al., 2014). Moreover, there are numerous studies that focus on the importance of pharmaceutical therapies to diminish neuroinflammation in CNS disorders by suppressing the ERK pathway (Jeong et al., 2019; Lim et al., 2019; Shao et al., 2013; Wang et al., 2010; Xia et al., 2015; Zhao et al., 2013). On the other hand, it has been reported that inhibition of microglial ERK attenuates the anti-inflammatory effects of anti-malarial agent, artesunate. Accordingly, it is suggested that ERK pathway might be required for anti-neuroinflammatory responses (Lee et al., 2012).

Supporting this conclusion, a large body of evidence have reported that time is a determinant factor for the activity of signaling molecules (Abbasnejad et al., 2019; Valledor et al., 2000). In accordance, Valledor et al. (2000) showed that peak time of ERK activity would determine how macrophages respond to proliferative or activating stimulants. Furthermore, our previous work on the effect of high-fat diet consumption on hippocampal MAPK signaling pathway, clearly demonstrated that JNK, P38 and ERK have a fluctuating pattern of activities when examined at different intervals after HFD consumption (Abbasnejad et al., 2019). Consistent with our results, a cell culture study also showed time dependency of ERK activity; however in shorter periods of time. This study showed that incubating of HP-1 cells with LPS, leads to activation of the ERK pathway after 60min, co-treatment of these LPS-stimulated cells with minocycline increases ERK activity at 30min but decayed after 60min. By this mechanism, it induced early suppression of induced chemokines and cytokines (Sun et al., 2015). Moreover, induction of inflammation with calcitonin gene-related peptides also resulted in a time-dependent increase in ERK phosphorylation pat-



tern up to 1h (Permpoonputtana et al., 2016). In addition, the increased ERK activity during the first hours of inflammation was also confirmed in a traumatic injury model. In this study, a robust rise in ERK phosphorylation level at 10h post-injury followed by a decrease after 24h were reported. The results also showed a rise in IL-10 protein level as a stimulatory factor for ERK activity at first hours and increased mitogen-activated protein kinase phosphatase-1 and phospho protein phosphatase 2A level as inhibitory factors for ERK activity from 24h (Szelenyi and Urso, 2012). In addition, an immunohistochemistry analysis for expression of p-ERK in the neurons of the superficial dorsal horn after ipsilateral spinal cord (L5) ligation indicated ERK activity peaked at 10min after the injury and had a reducing trend until 6h (however, at this time the number of p-ERK positive neurons is still higher than the control group). Although, in contrast to our results, this elevated activity was present in more than 3 weeks (Zhuang et al., 2005). Moreover, in a Freund's adjuvant (CFA) injection induced acute peripheral inflammation model, Zhang and colleagues (2014), showed that p-ERK expression level in the spinal cord dorsal horn started to increase at 2h, reached to maximum level after 24h and this increased level remained high 7days after induction of inflammation. It is noteworthy to mention that LPS treatment was done in a lowered serum media (10% vs 1% FBS). Therefore, the same lowering of serum was also done for control cells and as a result, we had different control groups for different time intervals, (having the same duration of serum deprivation as corresponding LPS group) and results of each time point was compared to its own control group. This could explain why, in our experiment, control cells also showed some variation in ERK activity when compared with other time points.

## Conclusion

Collectively, our results clearly showed that before judgment about the activity level of a molecule such as ERK, we should consider this point that, in which time point the assessment was done? If we couldn't detect any changes in the activity level of a molecule in a particular time point, it doesn't necessarily mean that a molecule is not responsible at all. In fact, activation of each element in a signaling pathway could vary in a time dependent manner.

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## Conflicts of interest

The authors declare that they have no conflict of interests.

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