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Ubiquitous hazardous metal lead induces TNF-α in human phagocytic THP-1 cells: Primary role of ERK 1/2

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ABSTRACT

Induction of tumor necrosis factor-α (TNF-α) in response to lead (Pb) exposure has been implicated in its immunotoxicity. However, the molecular mechanism by which Pb upregulates the level of TNF-α is wagely known. An attempt was therefore made to elucidate the mechanistic aspect of TNF-α induction, mainly focusing transcriptional and post transcriptional regulation via mitogen activated protein kinases (MAPKs) activation. We observed that exposure of Pb to human monocytic THP-1 cells resulted in significant enhanced production of TNF-α m-RNA and protein secretion. Moreover, the stability of TNF-α m-RNA was also increased as indicated by its half life. Notably, activation of ERK 1/2, p38 and JNK in Pb exposed THP-1 was also evident. Specific inhibitor of ERK1/2, PD 98059 caused significant inhibition in production and stability of TNF-α m-RNA. However, SB 203580 partially inhibited production and stability of TNF-α m-RNA. Interestingly, a combined exposure of these two inhibitors completely blocked modulation of TNF-α m-RNA. Data tends to suggest that expression and stability of TNF-α induction due to Pb exposure is mainly regulated through ERK. Briefly, these observations are useful in understanding some mechanistic aspects of proinflammatory and immunotoxicity of Pb, a globally acknowledged key environmental contaminant.

1. Introduction

Lead (Pb), a well known hazardous material, has been in use in innumerable products of industrial as well as domestic kinds since very long, thus resulting contamination of each compartment of environment and worldwide exposure to human of any life style. Earlier studies had established the negative impact on different biological systems towards Pb exposure, e.g., neurological, haematological, reproductive, etc., however immune system is one of the most sensitive among them. Pb had the ability to induce immunosuppression and reduce host resistance towards infectious disease. Phagocytic cells (macrophages and monocytes) have been known as a significant target during Pb mediated immunotoxicity [1]. Earlier research related to immunotoxicity of Pb had served to highlight that many host problems (e.g., neurological, cardiac, renal, reproductive) arising from exposure to Pb may ultimately trace back to Pb-induced changes in myelomonocytic-derived cell population. Pb impairs several aspects of phagocytic cell functions including adherence, chemotaxis [2,3], and clearance of intracellular pathogens [4]. Moreover, Pb has also been shown to inhibit nitric oxide production in both myeloid as well as myeloid suppressor cells [5,6].

The generation of inflammatory responses is a key event of host defense system. Monocytes play important role in, tightly regulated process, at least in part, via the secretion of proinflammatory cytokines, mainly tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6. TNF-α is one of the principal mediators of the inflammatory responses in mammals, transuding deferential signals that regulate activation, proliferation and apoptosis at cellular levels [7]. Earlier studies identified the capacity of Pb to increase production of various pro-inflammatory cytokine mainly TNF-α among phagocytic cells of both animal and human [8–12], While the production of TNF-α can be elevated following exposure to lead, the expression of the receptor for TNF-α was also increased during the *in vitro* exposure of human blood monocytes to $PbCl_2$ [13]. Therefore, the combined effect of elevated cytokine production by macrophages and of increased receptor expression would be expected to contribute

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to problematic inflammatory responses. In addition to this Pb-induced effect seem to be also involved in elevated production of the other major pro-inflammatory cytokines i.e. IL-1β and IL-6 [14,15].

Proinflammatory cytokines induction is tightly regulated act through receptor-dependent signaling cascades that diverge into multiple pathways, including each of the three mitogen-activated protein kinases (MAPK): extracellular-regulated kinase (ERK1/2), stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), and p38 [16]. While each of these signaling pathways contributes to the activation of gene transcription, their role in controlling gene expression at the level of mRNA stability is not well understood. Although the majority of studies suggest that mRNAs encoding for proinflammatory genes including, TNF-α, cyclooxygenase-2, IL-6, and IL-8 are stabilized upon activation of the p38 and ERK1/2 MAP kinase pathway [17–20]. These MAPK-mediated stabilization is dependent on AU-rich elements (ARE) sequences in the 3′-untranslated region (UTR) of respective genes, suggesting that AREs not only confer instability on mRNAs, but they also allow mRNA stabilization following activation of MAPK pathway [21]. Despite the suggested role of TNF-α in the pathogenesis of various inflammatory disease, the mechanisms by which Pb exposure increases TNF-α expression are not well understood. Pb had the capability to modulate the activity of several important signaling molecules including ERK1/2, p38 MAPK and JNK [22–25]. However, it is not known whether changes induced by Pb in these pathways can modulate TNF-α transcription and/or translation in phagocytic cells.

In view of the importance of TNF-α in inflammatory diseases and the potential for it to be upregulated by Pb, it is critical to understand how Pb induces expression of this cytokine at the molecular level. The purpose of this study was to test the hypothesis that Pb-induced activation of MAPKs mediates transcriptional and posttranscriptional upregulation of TNF-α expression. More specifically, the effects of Pb on ERK1/2, p38, and JNK1/2 activation, TNF-α m-RNA stability and TNF-α protein production were assessed in human monocytes THP-1 cells.

2. Materials and methods

2.1. Reagents

Cell culture reagents were from Life Technologies, Inc. (Grand Island, NY). Kinase inhibitors PD-98059 (2′-amino-3′-methoxy flavone) and SB-203580 (4-(4-fluophenyl)-2-(4 methylsulfinyl phenyl)-5-(4-pyridyl)1H-imidazole) were purchased from Calbiochem (San Diego, CA). Lead acetate, LPS (*E. coli* serotype 055:B5), Actinomycin D and β-actin antibody were from Sigma–Aldrich (St. Louis, MO). (α-^{32}P)dCTP was purchased from ICN Pharmaceuticals (Irvine, CA). Specific anti-phospho ERK (Thr202/Tyr204), anti-phospho JNK (Thr183/Tyr185), anti-phospho p38 (Thr180/Tyr182) anti-ERK, anti-JNK anti-p38 were obtained from Cell Signalling Technology (Beverly, MA) and were used in the ratio of 1:1000 in all experiments. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were used in ratio of 1:500 in all experiments. Ribonuclease Protection Assay (RPA) kit and reagents were purchased from BD pharmigen (San Diego, CA). The rest of the chemicals used in the study were of analytical grade of purity and procured locally.

2.2. Cell culture

The human promonocytic cell line THP-1 was purchased from National Centre for Cell Sciences, Pune, India. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 5% CO_2 incubator. Cells were maintained at a density of 5×10^5 to 1×10^6 cells/ml, and used in log-phase of growth between the ninth and eighteenth passages. These cells express various receptors that are found in normal monocytes and have been used as a model system for monocyte/macrophage biology [26].

Table 1
The sequence of primers for PCR.

Gene	Primer (5′–3′)	Length (bp)
TNF-α	F: GGCTCCAGGCGGTGCTTGTTC R: AGACGGCGATGCGGCTGATG	409
IL-1β	F: CTTCATCTTTGAAGAAGAACCTATCTTCTT R: AATTTTTGGGATCTACACTCTCCAGCTGTA	332
IL-6	F: GATGGATGCTTCCAATCTGGAT R: AGTTCTCCATAGAGAACAACATA	450
β-actin	F: CCCAAGGCCAACCGCGAGAAGAT R: GTCCCGGCCAGCCAGGTCCAG	219

F: forward; R: reverse.

2.3. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay

The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of the cells. The MTT-colorimetric monocyte mediated cytotoxicity assay, based upon the ability of living cells to reduce MTT into formazan by mitochondrial succinate dehydrogenase in viable cells. Twenty-four hours after cell seeding, cells were incubated with varying concentrations of Pb (0.1–100 μm) for 6 and 24 h at 37 °C. Following the removal of the exposure from each well, cells were washed in phosphate-buffered saline (PBS). The cells were then incubated in serum-free RPMI to which MTT (0.5 mg/ml) was added to each well (100 μl) and incubated for a further 4 h. Then the medium was removed and the cells were incubated for 15 min with 100 μl of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader (Synergy HT Biotek, USA). Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

2.4. RNA isolation from THP-1 cells for RT-PCR

Total RNA was extracted from cells culture using the TRIzol system (Sigma, USA), in accordance with the manufacturer's instructions. In brief, cDNA was prepared using RNA samples (3–5 μg) to which 1 μg oligo(dT)$_{18}$, 0.5 mM dNTP, and 200 U of Revert Aid^{TM} H Minus M-MuL V RT enzyme (MBI, Fermentas, USA) were added. The gene specific primers used were synthesized by are listed in Table 1. The PCR products identified by 1% agarose gel electrophoresis were analyzed using IS1000 image analysis system (Alpha Innotech, San Leandro, CA, USA). All samples were analyzed in triplicate.

2.5. Total cell lysate preparation

Following treatment to the cells, the medium was aspirated and the cells were washed twice with cold PBS (10 mM, pH 7.4). Ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, pH 7.4) was added to the plates, which were then placed over ice for 30 min and then the lysate was collected in a microfuge tube. The lysates was cleared by centrifugation at 14,000 × *g* for 15 min at 4 °C and

the supernatant (total cell lysate) was either used immediately or stored at −80 °C.

2.6. Western blotting

Western blotting was carried out according to method of Towbin et al. [27] with some modifications. Proteins (100 μg) were resolved on 10–12% SDS–PAGE and then electroblotted onto nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk and probed with different primary antibodies at dilutions recommended by the suppliers. Further immunoblots were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG using chemiluminiscence kit and visualized by Versa Doc Imaging System (Biorad, CA, USA). To quantify equal loading, membrane was reprobed with β-actin antibody. Data are presented as the relative density of protein bands normalized to β-actin. Densitometric measurements of the bands were done with digitalized scientific software program, UN-SCAN-IT, purchased from Silk Scientific Corporation (Orem, UT, USA).

2.7. Probe synthesis

The human TNF-α cDNA clone was purchased from American Type Culture Collection and grown in Luria–Bertani broth (Himedia, India) containing ampicillin (50 μg/ml; Sigma) or tetracycline (20 μg/ml; Sigma), respectively. Plasmid containing the TNF-α was isolated using the QIAprep spin miniprep kit (Qiagen, Valencia, CA) and subsequently digested with restriction endonucleases (New England Biolabs, Beverly, MA) HindIII and AvaI Digestion of the TNF-α cDNA resulted in a 578-bp fragment containing 450 bp of the TNF-α coding region as well as 128 bp of the 3′-UTR. Fragments were visualized with ethidium bromide after electrophoresis of the digests into 1.2% agarose gels. The 578-bp DNA fragment was excised and purified using the QIAquick gel extraction kit (Qiagen). Approximately 25 ng of the TNF-α fragments were resuspended in sterile water for use in [α-^{32}P]dCTP random primer labeling reactions employing the Rediprime II kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). A 316-bp human GAPDH DNA probe (Ambion, Inc., Austin, TX) was also randomly prime labeled using the Rediprime II kit to detect GAPDH mRNA that served both as a mRNA stability control and loading control.

2.8. Northern blot analysis

Total RNA from THP-1 cells was extracted using the TRIzol reagent (Gibco Life Technologies) as per manufacturer's protocol. Following extraction, total RNA samples (10–15 μg) were electrophoresed into 1% agarose/formaldehyde gels at 50 V for 1.5 h and then transferred to positively charged nylon membranes up to for 3 h. Transferred RNA was than cross-linked to membranes using a UV cross-linker (Bio-Rad, Hercules, CA). Membranes were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS for 30–60 min, after which they were pre-hybridized at 42 °C for 3 h with salmon testes DNA (Sigma) in a pre-hybridization solution of 50% formamide (Sigma) and 10% SDS (Sigma). Now radiolabeled probes were then added to the hybridization buffer overnight at 42 °C. After hybridization, blots were again rinsed with 2× SSC (with 1% SDS) for 15 min at room temperature, and then washed once with 0.1× SSC (with 1% SDS) for 30 min at 60 °C. Blots were then dried briefly and TNF-α and GAPDH mRNA were visualized by autoradiography and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and corrected by reference to the corresponding GAPDH reading to compensate for slight variation in loading.

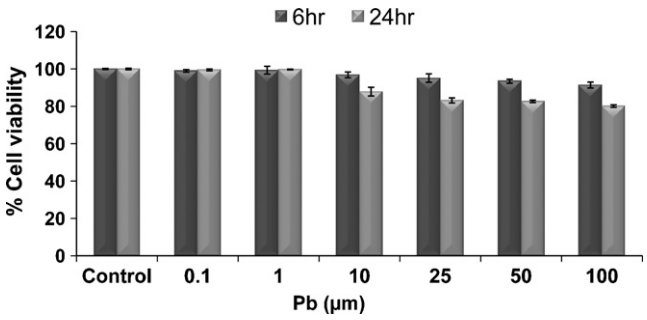

Fig. 1. Effect of Pb exposure on cellular viability. THP-1 cells were exposed to different concentrations (0.1–100 μM) of Pb for 6 and 24 h. Cellular viability was measured by MTT assay. Results were calculated by averaging three independent experiments.

2.9. Ribonuclease protection assay

Total RNA from THP-1 cells was extracted using the TRIzol reagent (Gibco Life Technologies) as per manufacturer's protocol. For mRNA stability experiments, cells were further cultured in the presence or absence of Actinomycin D (10 μg/ml) for the indicated times following Pb treatment before RNA was isolated. For some experiments THP-1cells were pretreated with either 20 μM SB203580 or 25 μM PD98059 before Pb stimulation and RNA isolation. Previously synthesized riboprobe for TNF-α was used and RPAs were carried out following the manufacturer's instructions. Samples were run on 5% sequencing gels, dried, and autoradiographed (Molecular Dynamics, Sunnyvale, CA).

2.10. TNF-α protein quantification

The extracellular medium was centrifuged at 250 × *g* for 10 min and the supernatant was stored at −20 °C. For determination of intracellular TNF-α content, cells were lysed, centrifuged and the supernatant was stored at −20 °C. TNF-α was analyzed using a commercial ELISA kit (Biosource, Camarillo, CA).

2.11. Statistical analysis

The data were analyzed to obtain mean values and standard deviation for all treated and vehicle control samples, which were subjected to statistical comparison using student-*t*-test, $p < 0.05$ was considered as significant.

3. Results

3.1. Pb treatment alters cellular viability

The human monocytic cells THP-1 were exposed to increasing concentration of Pb (0.1–100 μM) for different time periods (6 and 24 h). The results showed that the exposure concentration of 100 μM or less had only mild effect on cellular viability i.e. more than 80% were found to be live in all time periods (Fig. 1). Based on the above results we used exposure doses less than 100 μM throughout the study.

3.2. Pb treatment upregulates m-RNA levels of TNF-α and other pro-inflammatory cytokines

We initially analyzed the m-RNA levels of TNF-α and other pro-inflammatory cytokines (IL-1β and IL-6) by RT-PCR. As indicated in Fig. 2A, Pb treatment for 24 h enhanced TNF-α, IL-1β and IL-6 m-RNA expression levels. Similar results were observed when LPS (200 ng/ml) was used, as a typical pro-inflammatory agent. Further analysis of time course of TNF-α m-RNA induction in Pb

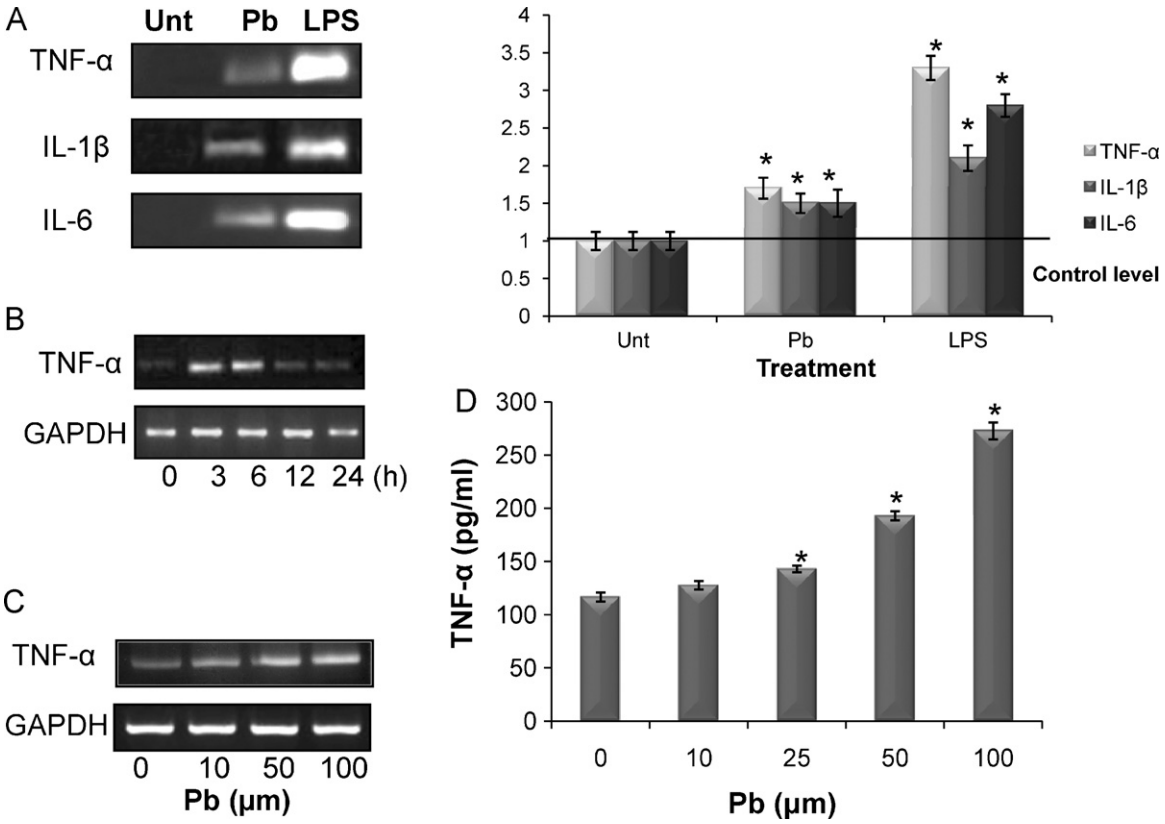

Fig. 2. (A) THP-1 cells were treated by Pb (50 μM) and LPS (200 ng/ml) for indicated time. m-RNA levels of TNF-α, IL-1β and IL-6 were than analyzed by RT-PCR. TNF-α expression in response to Pb in THP-1 cells (B) Time dependent (C) and dose dependent. (D) TNF-α secretion analyzed by ELISA. All the data are representative of three independent experiments. Asterisks indicate significant changes as compared with the control (*$P < 0.05$).

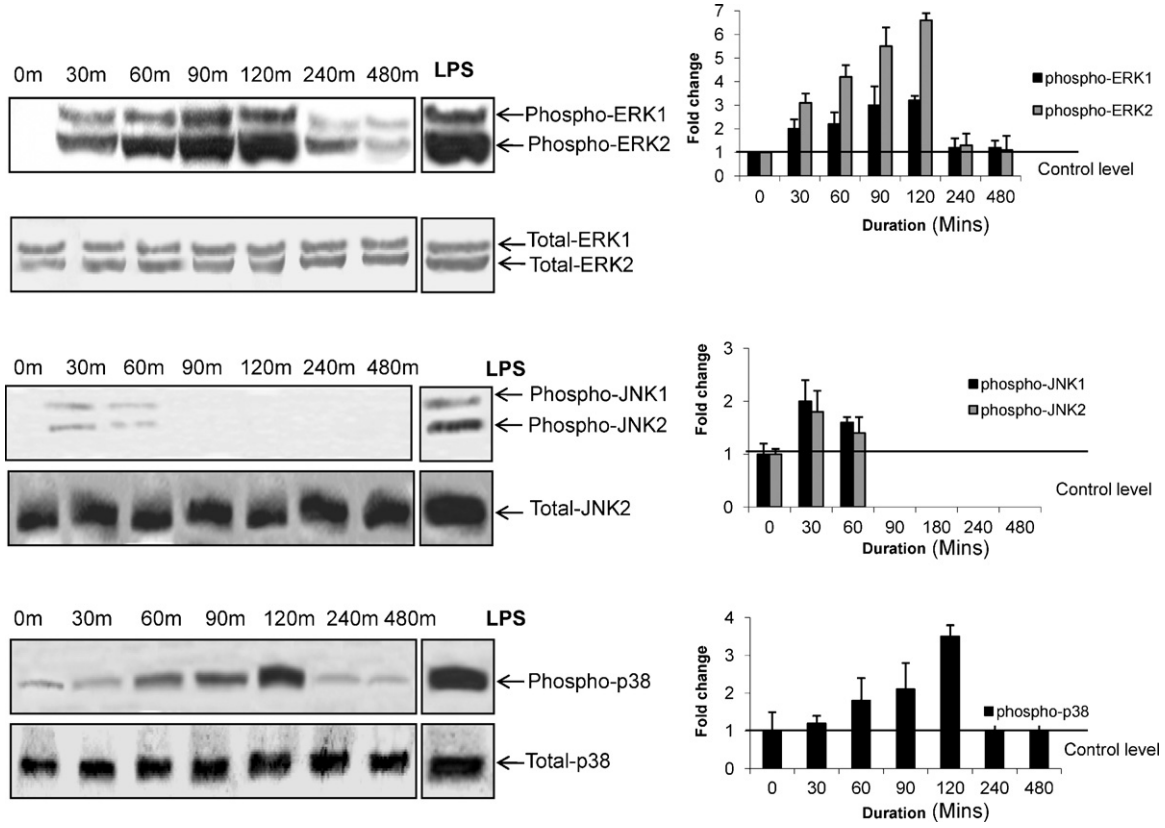

Fig. 3. Pb induces the phosphorylation of (A) ERK1/2, (B) JNK, and (C) p38 in THP-1 cells. THP-1 cells were treated with Pb (50 μM) for 30–480 min. Cell lysates were separated by SDS–PAGE and immunoblotted with ERK1/2, Phospho ERK1/2 (Thr202/Tyr204), JNK, Phospho-JNK (Thr183/Tyr185), p38 and Phospho-p38 (Thr180/Tyr182) antibodies.

exposed monocytes indicated that exposure period range 3–6 h was enough to get a maximal increase, where 12 h exposure had shown less decrease, however longer exposure periods (>12 h) lead to a diminished upregulation (Fig. 2B). Furthermore, we had also analyzed the dose–response of TNF-α m-RNA upregulation by Pb. As clearly shown in Fig. 2 C, the dose of 10–100 μM increased TNF-α m-RNA level in a concentration dependent manner. On the contrary, low doses of Pb (less than 10 μM) had shown no significant effect on TNF-α m-RNA level (data not shown). Similar observation was recorded in TNF-α production at protein levels also (Fig. 2D).

3.3. Pb treatment activates various MAPKs in THP-1 monocytes

We analyzed the possible signaling mechanism that could be involved in Pb mediated TNF-α regulation. For this, we studied the activation of the three major types of MAPKs, i.e., ERK1/2, p38 and JNK. Primarily it was found that Pb constituently activated the ERK1/2 pathway in THP-1 cells. As evident from the western blotting experiments, Pb clearly enhanced ERK1/2 phosphorylation highest at 120 min. The phosphorylation pattern clearly indicated a gradual increase in phosphorylation over the time up to 120 min, however the phosphorylation tends to decreased onwards i.e. 240 min (Fig. 3A). The exposure of Pb modified the levels of total JNK but a slight increase in phosporylated form of JNK at 30 min, which was abolished (Fig. 3B) with time. Interestingly, the phosphorylation pattern of p38 was also similar to that of ERK1/2 as apparent in Fig. 3C. In all the cases 1 h LPS exposure (200 ng/ml) was used as a positive control, which displayed a marked activation of all the three MAPK's.

3.4. Inhibition of ERK phosphorylation decreases TNF-α m-RNA levels

To further determine the role of ERK1/2 and p38 phosphorylation in Pb mediated upregulation of TNF-α m-RNA levels, we investigated the effects of MAPK inhibitors namely PD-98059 for ERK1/2 and SB-203580 for p38. The inhibition of ERK1/2 phosphorylation by applying PD-98059 (25 μM) in Pb-treated monocytes clearly abolished the upregulation of TNF-α m-RNA. Moreover, very less or non-significant inhibition of TNF-α m-RNA was also observed in presence of SB-203580 (20 μM) (Fig. 4).) In order to specify RT-PCR results, we performed Northern blot analysis, which showed significant inhibition of TNF-α m-RNA in presence of ERK1/2 inhibitor (PD 98059), however p38 inhibitor showed less effect (Fig. 5A and B). We further analyzed the combinatorial effect of both MAPK inhibitors in combination on TNF-α m-RNA level; which showed nearly complete attenuation of TNF-α m-RNA level (Fig. 6). However, no loss of human monocytic cells viability was observed at the concentrations of inhibitors used.

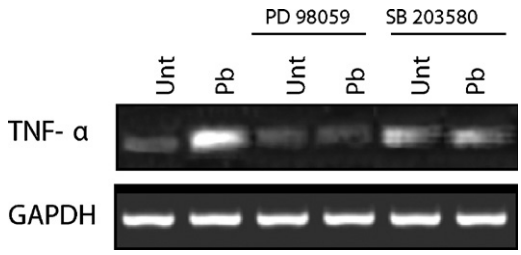

Fig. 4. THP-1 cells pretreated for 1 h with 20 μM SB 203580, 25 μM PD 98059, followed by Pb (50 μM) for indicated time. m-RNA levels of TNF-α and GAPDH were detected by RT-PCR. Data are representative of three independent experiments.

3.5. Effect of MAPK inhibitors on TNF-α production

The different MAPK inhibitors were also used to determine whether activation of ERK1/2 or p38 modulated the Pb induced TNF-α protein production. Cells were incubated with Pb alone and in presence of inhibitors (PD 98059 and SB 203580) for 24 h and TNF-α secretion was assessed by ELISA. Pb (50 μM) induced TNF-α production was inhibited in a concentration dependent fashion at 24 h by both inhibitors (Fig. 7A and B). Moreover, in the presence

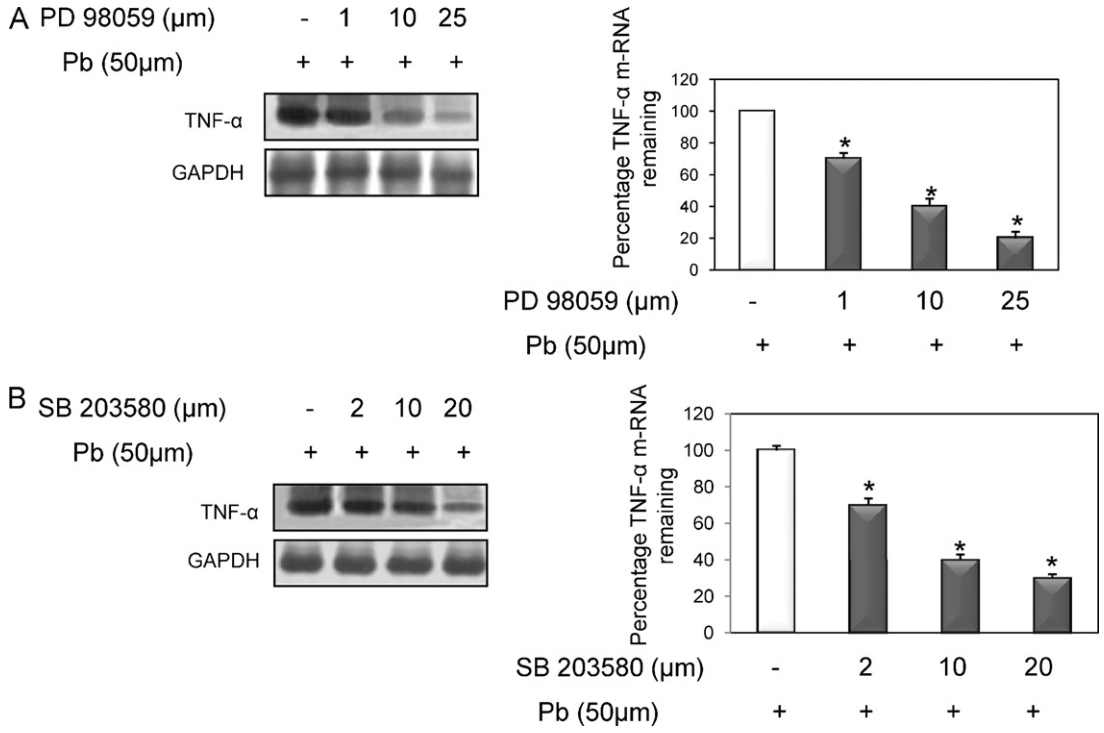

Fig. 5. Effect of different doses of (A) PD 98059 (1–25 μM) and (B) SB 203580 (2–20 μM) on TNF-α induction, analyzed by Northern blot in THP-1 cells. Data are representative of three independent experiments. Asterisks indicate significant changes as compared with the control (*$P < 0.05$).

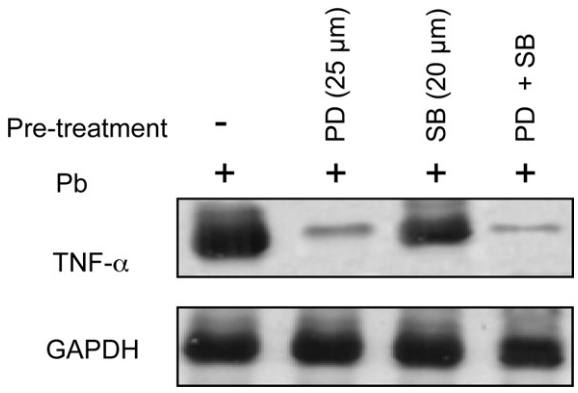

Fig. 6. THP-1 cells were pretreated for 1 h with 20 μM SB 203580, 25 μM PD 98059 or combination of both, followed by Pb (50 μM) for indicated time. Cellular RNA was isolated and analyzed. Data are representative of three independent experiments.

of JNK inhibitor SP 600125 (10 μM), LPS induced TNF-α production was significantly reduced whereas Pb induced TNF-α production was not affected (data not shown).

3.6. Effect of MAPK inhibitors on Pb-induced TNF-α m-RNA stability

In order to find out the exact mechanism that how Pb increases the TNF-α m-RNA levels it was essential to perform the studies that reflect the effect of Pb on TNF-α m-RNA stability. For, this TNF-α expression was induced by adding LPS (1 μg/ml) in culture for 3 h and then stability of its m-RNA was assessed in presence or absence of inhibitors. m-RNA was isolated at different time intervals (60, 120 and 240 min) after treatment with Pb alone or Pb + MAPK inhibitors in presence of Actinomycin D (10 μg/ml),. Actinomycin D treatment suppressed TNF-α m-RNA expression at each time interval, resulting in a TNF-α m-RNA half life of 49 min (Fig. 8A). Expression of housekeeping GAPDH m-RNA was not changed by Actinomycin D treatment. The presence of Pb increased TNF-α m-RNA stability, resulting in a TNF-α m-RNA half life up to 240 min. When the ERK1/2 inhibitor PD 98059 (25 μM) was co-incubated with Actinomycin D and without Pb treatment, m-RNA half life decreased from 49 to 15 min. The result referred to suggests that ERK1/2 is required to stabilize TNF-α m-RNA in non Pb treated cells. Similarly, Pb mediated TNF-α m-RNA half life (up to 240 min) was also reduced to 27 min in the presence of ERK1/2 inhibitor. Moreover, when we compared the percentage of remaining TNF-α m-RNA both in absence and presence of ERK1/2 inhibitor, we observed clear involvement of ERK1/2 in Pb induced TNF-α m-RNA stability. Similarly, we employed SB203580 (20 μM) to investigate whether p38 pathway is involved in Pb induced TNF-α m-RNA stabilization. Actinomycin D treatment resulted in a TNF-α m-RNA half life of 49 min (Fig. 8B). When the inhibitor was co-incubated with Actinomycin D and without Pb treatment, TNF-α m-RNA half life was reduced to 36 min. These results indicated that p38 activation could also be involved in stabilizing the TNF-α m-RNA. Pb (50 μM) increased the TNF-α m-RNA half life up to 240 min. This increased half life was reduced to 62 min in presence of SB 203580. The above results clearly suggested that Pb induced TNF-α m-RNA stabilization was also significantly regulated by p38.

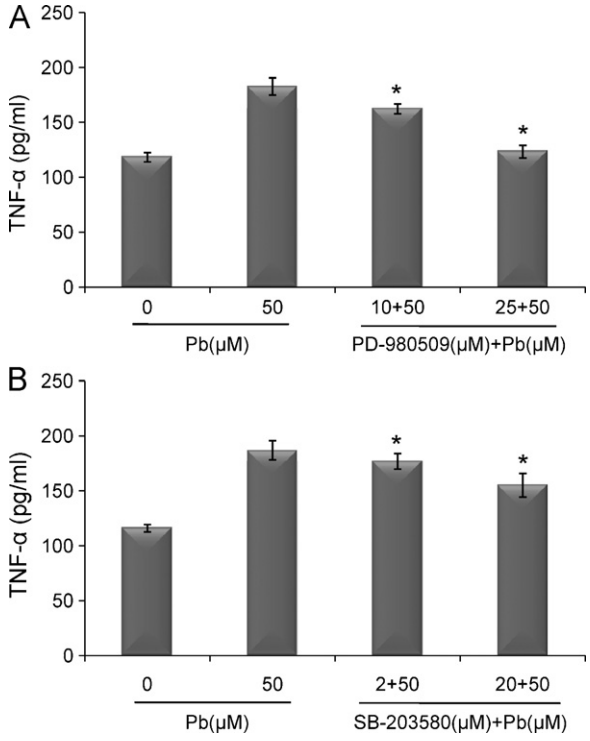

Fig. 7. Effect of ERK1/2 and p38 inhibition on TNF-α release in THP-1 cells. (A) THP-1 cells were pretreated for 1 h with PD 98059 (25 μM) before 24 h incubation with Pb (50 μM). TNF-α levels were measured by ELISA. Data are representative of three independent experiments. (B) Similar conditions were applied in presence of SB 203580 (20 μM). Asterisks indicate significant changes as compared with the Pb (50 μM) (*$P < 0.05$).

4. Discussion

It is a well known that pleiotropic cytokines such as TNF-α are produced predominantly by activated monocytes, macrophages and lymphocytes and plays a central role in inflammation. In the present study, we found significant induction of TNF-α both at m-RNA and protein levels from monocytes exposed to Pb. We had also observed higher m-RNA levels of other proinflammatory cytokines i.e. IL-1β and IL-6. Our results are in clear agreement with the previous reports that have reported significant induction in different proinflammatory cytokine levels after Pb exposure either *in vitro* or *in vivo* [28–31].

The MAPK signal transduction pathways are critical regulators for inducing inflammatory gene expression after exposure to different types of mitogens. Moreover, MAPKs could directly target the RNA polymerase II complex to bring about transcriptional activation of proinflammatory cytokines such as TNF-α at the promoter level [32,33]. These mechanisms are fundamental in the initiation of inflammatory responses. We have observed that ERK1/2 and p38 were significantly activated in Pb exposed monocytes and that the MAPKs mediated induction of TNF-α is mainly via ERK1/2 mediated pathway. To further prove the role of activated ERK1/2 and p38 in induction of TNF-α m-RNA, we suppressed the phosphorylation of ERK1/2 by using PD-98059, which specifically block the phosphorylation of ERK1/2, by inhibiting phosphorylation of MEK1/2. We found that inhibition of ERK1/2 results in significant ablation of TNF-α m-RNA, whereas inhibition of p38 by specific inhibitor SB-203580 also substantially decreases the TNF-α m-RNA. When both of the inhibitors were used in combination, TNF-α m-RNA levels were completely diminished. Based on the above intriguing facts it is evident that ERK mainly regulate TNF-α production at transcriptional levels in Pb exposed monocytes. However we did not deny the fact, p38 also played a significant role in regulating the TNF-α production in Pb exposed monocytes. Our study is in clear coordination with other studies that have shown ERK1/2 dependent induction of TNF-α at both transcriptional and post-transcriptional levels [34–36]. It has also gained strength from another report, which has established the role of other MAPKs i.e.

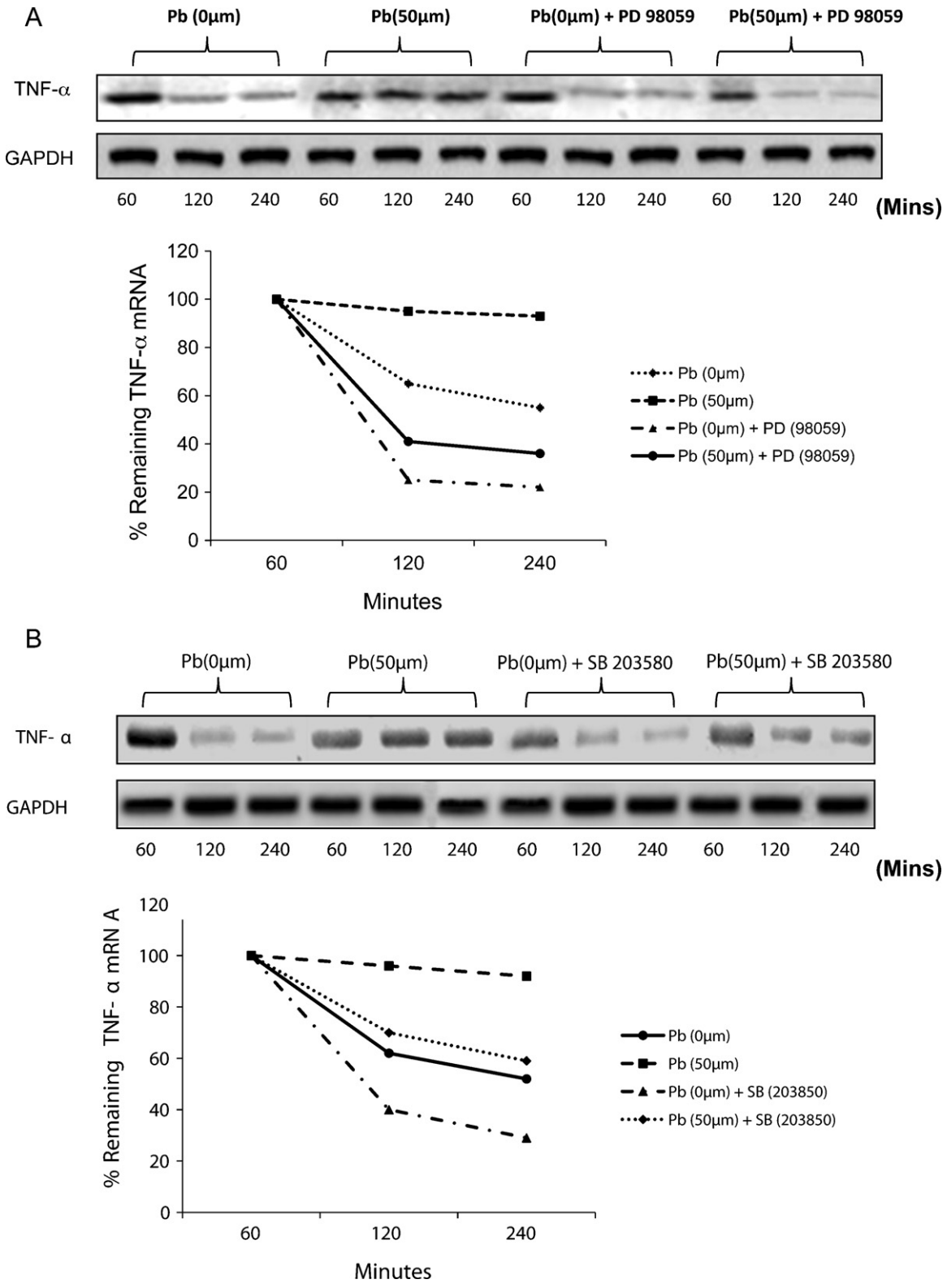

Fig. 8. (A) Effect of ERK1/2 inhibition on Pb induced TNF-α m-RNA stability in THP-1 cells. Cells were treated either with Pb (0 μM), Pb (50 μM) or in combination with PD 98059 (20 μM) in presence of Actinomycin D (10 μg/ml) for indicated times. Total RNA was analyzed by RNase protection assay. (a) TNF-α mRNA blot from RNase protection assay. (b) Relative TNF-α mRNA levels after normalization with GAPDH gene expression determined by densitometry of blots from RNase protection assay. Data are representative of three independent experiments. (B) Effect of p38 inhibition on Pb induced TNF-α m-RNA stability in THP-1 cells. Cells were treated either with Pb (0 μM), Pb (50 μM) or in combination with SB 203580 (20 μM) in presence of Actinomycin D (10 μg/ml) for indicated times. Total RNA was analyzed by RNase protection assay. (a) TNF-α mRNA blot from RNase protection assay. (b) Relative TNF-α mRNA levels after normalization with GAPDH gene expression determined by densitometry of blots from RNase protection assay. Data are representative of three independent experiments.

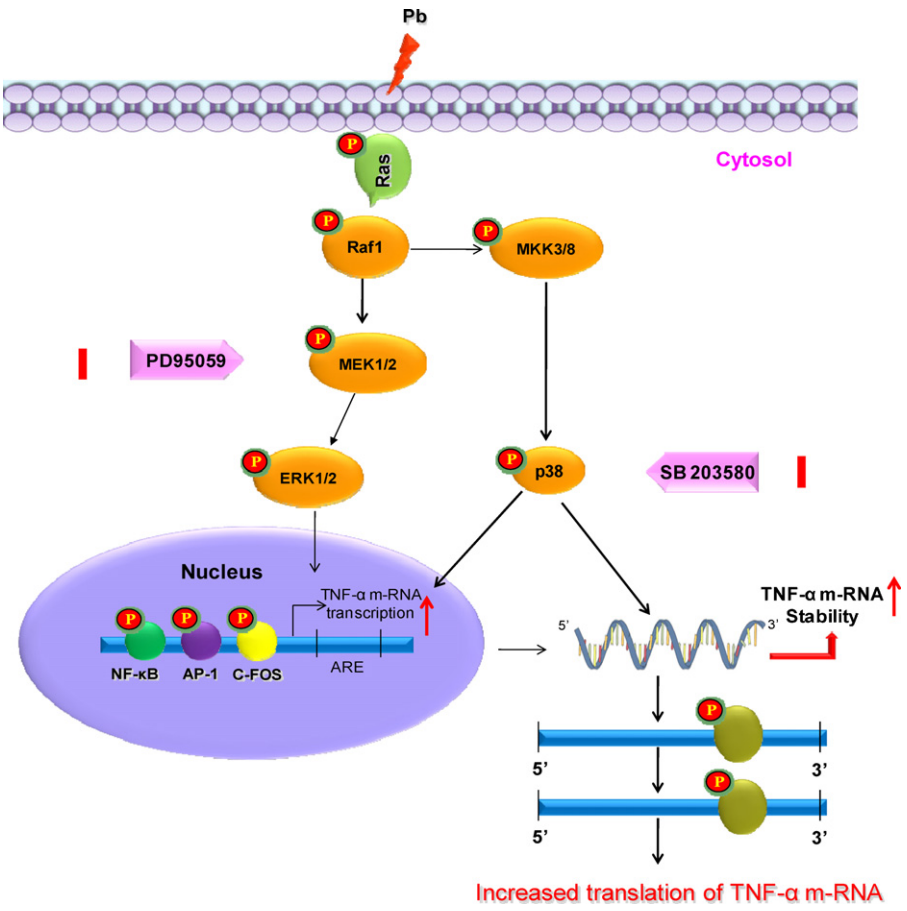

Fig. 9. Hypothetical proposed mechanism for induction of TNF-α by Pb in human monocytes.

p38 and JNK, in regulating TNF-α production at both translational and post-translational levels [37–40].

Investigations into the post-transcriptional control of TNF-α have focused on the importance of ARE present in the UTR of TNF-α RNA as a critical region involved in the regulation of TNF-α message stability and translation [41,42]. In unstimulated cells, the TNF-α message is unstable and translationally repressed, preventing TNF-α protein production [43]. In response to mitogen mediated stimulation, such as by LPS, the TNF-α message is stabilized and translation is de-repressed, allowing for the rapid production of TNF-α [44,45]. In the present study we observed increased stability of TNF-α m-RNA after Pb exposure. Moreover the TNF-α m-RNA stability was dependent on ERK1/2 activation, because blocking of the ERK phosphorylation resulted in decreased stability of TNF-α m-RNA. Furthermore, the TNF-α m-RNA stability tends to decrease further when ERK and p38 inhibitors were used in combination with Pb. Our results demonstrated the inhibition or complete ablation of TNF-α m-RNA stability either by ERK1/2 alone or by combination of both inhibitors, which is consistent with the earlier reports [46,47]. In addition, nuclear export of TNF-α m-RNA is also a regulated event requiring ARE as well as an intact Tpl2/ERK signaling pathway. Unlike the Raf-1/ERK pathway, which mainly responds to mitogens, Tpl2 signaling is activated by TLR or proinflammatory cytokine stimulation [48]. Although, precise mechanism involved in the induction of TNF-α levels by Pb is not yet known, ERK1/2, which is getting activated by Pb, has been shown to regulate TNF-α production in several ways. It has been reported that the stimulation of macrophages by LPS results in increased ERK1/2 phosphorylation as well as increased nuclear transport of TNF-α m-RNA, which in turn enhances TNF-α production [49]. It has also been shown recently that selective blocking of the ERK1/2 phosphorylation (at MEK1/2) does not have significant effect on the pre-TNF-α production (an immature form of TNF-α), but secretion of the mature TNF-α was reduced markedly from macrophages exposed to LPS [50]. Thus ERK1/2 appears to play role in the regulatory mechanisms that are responsible for the maturation of TNF-α prior to its secretion. Since inhibition of ERK1/2 and p38 decreases the TNF-α secretion in Pb exposed monocytic cells, it is be possible that a similar mechanism operates through Pb, in this case (Fig. 9).

In summary, we had mainly focussed on the activation of MAPKs and their regulatory effect on the TNF-α production at transcriptional level after Pb exposure in human monocytic cells. Several studies have suggested regulatory role of MAPKs in proinflammatory cytokines production after Pb exposure [28,30] in other cell systems (glioma and hippocampal) rather than phagocytic cells. Moreover, Guo et al. [13] have provided initial clues for the transcriptional and posttranscriptional regulation of TNF-α production from phagocytic cells but the study lacks information regarding the regulatory role of MAPKs in TNF-α production. We for the first time have shown the regulatory role of MAPK's in TNF-α production in Pb exposed human phagocytic cells. Clearly, studies are required to increase our understanding about the role of other important signaling molecules (MAPKK's and MAPKKK's) and transcription factors (Nf-κB, EGR-1, c-Fos etc.) in ERK1/2 mediated induction of TNF-α production in Pb exposed monocytic cells to completely dissect the molecular mechanism of TNF-α induction at both transcriptional and posttranscriptional levels.

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