

Effect of nitric oxide donor and gamma irradiation on modifications of ERK and JNK in murine peritoneal macrophages

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Abstract Mitogen activated protein kinases (MAPKs) play an important role in activation, differentiation and proliferation of macrophages. Macrophages, upon activation, produce large amounts of nitric oxide that inhibit the growth of variety of microorganisms and tumor cells. This nitric oxide which is known to interfere with tyrosine phosphorylation may result in changes in the pattern of activation of MAPKs. In a previous study we have found that tyrosine phosphorylation of MAPKs was completely abolished in the presence of nitric oxide donor and radiation but this did not affect the function of macrophages. In this study the other post translational modifications namely nitration and ubiquitination of JNK and ERK have been looked at. Both ERK and JNK were found to be nitrated. However, there was no increase in ubiquitination of ERK and JNK, indicating that ubiquitination, in this case was not a natural consequence of nitration and may serve in signaling. Additionally, when the nitration was extensive, phosphorylation was also inhibited. The activation of substrates of ERK and JNK were looked at to determine the consequences of such modifications. Inhibition of phosphorylation and extensive nitration of JNK did not prevent activation of its substrate, *c-jun*. This study indicates that ERK and JNK may be under regulation by different type of modifications in macrophages.

Keywords Sodium Nitroprusside (SNP) · Radiation, Mitogen Activated Protein Kinase (MAPK) · Macrophages and post translational modifications

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Introduction

Signal transduction by mitogen activated protein kinases (MAPKs) is known to involve sequential phosphorylation of tyrosines or serines of the proteins (Martin-Blanco 2000). The last effector in the cascade is a transcription factor, which ultimately binds to DNA and leads to the expression of target genes. The MAP kinase family is composed of the ERK 1/2, p38 and SAPK/JNK pathways and is important for a wide spectrum of cell functions. Although distinct in their activation, there is considerable co-operation between these kinases and many substrates are shared between the pathways (Cobb 1999).

In macrophages, MAPK pathways are activated upon binding of growth factors and inflammatory cytokines to specific receptors and are essential in mediating important cell functions such as proliferation, differentiation (Hu et al. 2000; Das et al. 2000; Valledor et al. 2000), apoptosis (Mohr et al. 1998), cytokine biosynthesis (Kurosawa et al. 2000; Geppert et al. 1994; Kovalovsky et al. 2000), expression of cyclooxygenase-2 and iNOS (Chan et al. 1999; Chen et al. 1999) and phagocytosis (Kugler et al. 1997).

When radiotherapy is delivered to malignant cells, macrophages being radio resistant survive, get activated by radiation and regulate inflammatory and immunological responses. Activated macrophages also produce large amounts of nitric oxide that inhibits the growth of wide variety of tumor cells and microorganisms (MacMicking et al. 1997), and has also been shown to induce apoptosis in macrophages themselves (Albina et al. 1993; Messmer et al. 1995). The activation of MAPKs has been reported to be involved in the apoptotic response to nitric oxide (NO) in macrophage-derived cell lines and MEK inhibitor, inhibitor of upstream activator of ERK, has been shown to protect against cell death after macrophage activation (Mohr et al.

1998). However many reports have shown the protective effect of NO on macrophages (Yoshioka et al. 2006a, b) and other cells (Davis et al. 2000; Li et al. 1997; Zech et al. 2003). These dual effects of NO on macrophages have been imparted to different concentrations of NO. Low concentrations of NO have been shown to be protective while high concentrations have been shown to be cytotoxic. These varied effects of NO are mediated via activation or inhibition of signaling components involved, MAPKs being one of them.

With a better understanding of cellular stress response, it has become evident that the catalytic modules consisting of kinases that mediate the activation of downstream effector components are subject to multiple layers of regulation. Many post translation modifications like nitrosylation, nitration and even ubiquitination of signaling proteins seem to play important role in transducing the signal (Di stasi et al. 1999; Lane et al. 2001; Schnell and Hicke 2003; Ischiropoulos 2003). These modifications seem to act by altering the protein stability, localization or protein–protein association.

Nitric oxide is known to interfere with tyrosine phosphorylation and can either diminish the efficacy of a protein as a substrate for tyrosine kinases (Gow et al. 1996; Kong et al. 1996) or lead to their activation by increased phosphorylation directly (Monteiro et al. 2000; Oliveira et al. 2003; Bernabe et al. 2001) or by inactivation of phosphatases (Takakura et al. 1997). Other modifications like S-nitrosylation (Deora et al. 2000; Mondoro et al. 1997) and nitration (Balafanova et al. 2002; Cassina et al. 2000; Vadseth et al. 2004) may also be involved. Nitrated proteins have also been shown to be preferentially degraded via ubiquitin mediated pathways (Souza et al. 2003), hence determining the stability of protein in a cell.

Most of the studies regarding tyrosine nitration and other biological effects are derived from results either by treating macrophages with alkaline solutions of chemically synthesized peroxyxynitrite, NO donor or by activating macrophages with LPS/IFN- γ which themselves induce many signaling pathways. Simultaneous treatment with NO donor and radiation would be a better approximation in terms of generation of NO and reactive oxygen species together in macrophages. In this study we have used a small exposure to NO donor and 2 Gy of gamma radiation dose to simulate the early activation stage of simultaneous NO and ROS production in macrophages while avoiding LPS/IFN γ like inducers.

In our earlier study we had observed that when the cells were exposed to both radiation and a nitric oxide donor, phosphorylation of MAPKs was inhibited. The interesting aspect of this study was the fact that the macrophages were functionally unaffected in spite of the inhibition of MAPK phosphorylation (Narang and Krishna 2008). Since the

phosphatases were not significantly activated and the total amount of the kinases had not changed, it was of interest to look at the other post translational modification of these kinases. In this study the extent of these modifications in immunoprecipitated kinases (phosphorylation, nitration and ubiquitination) and the subsequent activation of the transcription factors has been looked at in peritoneal macrophages.

Material and methods

Animals

Male Swiss mice (4–6 weeks) were maintained on a standard laboratory diet with water ad libitum. Animals were reared in polypropylene cages in air-conditioned ($24 \pm 2^\circ\text{C}$) rooms with a 12-hourly dark and light schedule. Mice used in the present study were a part of conventional inbred colony of Swiss mice maintained at the animal house facility of Bhabha Atomic Research Centre.

All experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on use of animals in scientific research.

Isolation of PEC

Resident peritoneal exudates cells (PEC) were used as source of macrophages. PEC were isolated from animals by washing out the peritoneal cavity. In brief, RPMI 1640 (Sigma) was injected into peritoneal cavity of mice. After gentle massage of the abdomen, the media was withdrawn with the help of a syringe. The cells were then pelleted by centrifugation at 2500 rpm for 6 min, resuspended in RPMI 1640 medium at concentration of 5×10^6 cells/ml.

Treatment of PEC

The cells were divided into four treatment groups which were treated as follows: (a) Controls were untreated. (b) Cells were irradiated (2 Gy, dose rate 0.1 Gy/s, gamma cell 220, AECL, Canada) and lysed after 5 or 30 min. (c) One group of cells was treated with SNP (1 mM), one set was lysed after 60 min; two sets were irradiated after 30 min and lysed after 5 or 30 min respectively. Lysates were either directly processed for SDS-PAGE or were immunoprecipitated.

Immunoprecipitation

Treated or untreated cells (5×10^6) were lysed in cell lysis buffer by using anti rabbit anti-ERK (Sigma) or anti rabbit

anti-JNK (Sigma) using an immunoprecipitation kit (Sigma). Briefly lysates with protease inhibitors were centrifuged and the supernatants were incubated with anti-ERK or anti-JNK in immunoprecipitation (IP) buffer overnight at 4°C with gentle mixing. Protein A Sepharose beads were added and tubes were again incubated for 3 to 4 h at 4°C with gentle mixing. Different concentrations of IP buffer were used for washing the beads to remove unprecipitated protein. Samples were boiled for 10 min in SDS loading buffer to isolate the immunoprecipitated complex, eluent was collected and directly loaded onto gel for immunoblotting.

SDS-PAGE and Western blot analysis

For SDS-PAGE, 1×10^6 cells were lysed by boiling in SDS cell lysis buffer [10% glycerol, 2% SDS, 100 mM DTT, 0.1% bromophenol blue and 50 mM Tris-HCl (pH 6.8)] for 10 min. Lysates or immunoprecipitates obtained from these lysates as above were then loaded on to 8% SDS polyacrylamide gel electrophoresis followed by electroblotting onto nitrocellulose membrane (Amersham). After blocking of non-specific binding with 5% BSA (Sigma), the membranes were probed with the following antibodies. ERK immunoblot was probed with anti mouse anti ERK, anti mouse anti-phosphoERK (Sigma), anti mouse anti-nitrotyrosine, anti mouse anti-ubiquitin (Chemicon). JNK immunoblot was probed with anti mouse anti JNK, anti mouse anti-phosphoJNK (Sigma), anti mouse anti-nitrotyrosine and anti mouse anti-ubiquitin. The immunoblot of cell lysates were probed with anti rabbit phospho *c-jun* (cell signaling) and anti phospho Elk (cell signaling). This was followed by incubation with horseradish peroxidase (HRP) conjugated secondary anti mouse antibody (Roche Molecular Biochemicals, Germany). The secondary antibody was detected using BM Chemiluminescence Western Blotting Kit (Roche Molecular Bio Chemicals, Germany). The band intensity was quantified by software Gelquant (version 2.7.0 DNR Bioimaging Systems Ltd.).

Splenic lymphocytes treatment

Splenic lymphocytes were isolated by making a cell suspension by crushing the spleen on a sieve. The cell suspension was taken as splenic lymphocytes after RBC lysis. Splenic lymphocytes were treated with SNP (400 μ M) and or irradiation (2 Gy) for time periods as given in figures. After treatment the cells were lysed, processed for immunoblotting and probed with anti-ERK, anti-phosphoERK or anti-phosphoJNK.

All the experiments were carried out three times independently.

Results

Kinetics of IR and/or SNP induced MAPK activation, nitration and ubiquitination

Figure 1 shows the blots and histograms of tyrosine phosphorylation, nitration and ubiquitination of ERK after irradiation and SNP treatment. Tyrosine phosphorylation of ERK was induced as early as 5 min (Fig. 1, lane 2). This increase was transient since at 30 min there was a decline (lane 3). Following SNP treatment alone ERK was partially activated. However extensive irradiation induced phosphorylation observed at 5 min was inhibited with SNP treatment (compare lanes 2 and 5, Fig. 1). At 30 min the phosphorylation recovered but was not as extensive as with radiation alone (lane 6). Tyrosine nitration of ERK was almost absent

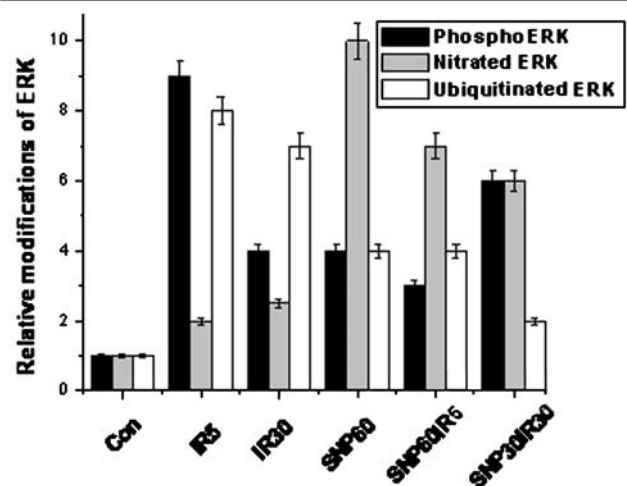
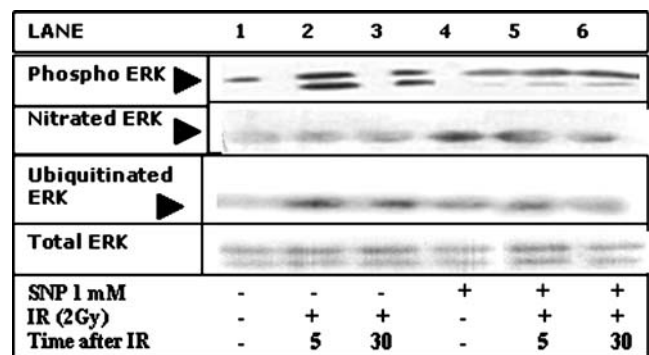


Fig. 1 Effect of SNP and/or IR treatment on tyrosine phosphorylation, tyrosine nitration and ubiquitination of ERK1/2: PEC were irradiated (2 Gy) and lysed after 5 and 30 min in the absence or presence of SNP (1 mM, 60 min). The total ERK was immunoprecipitated from the lysates with anti rabbit anti ERK followed by immunoblotting. Membranes were then probed by anti mouse anti ERK, anti phospho-ERK, anti-nitrotyrosine or anti-ubiquitin. **a** One of three resulting hybridization images of each is shown here. **b** Histogram representing relative intensities of phospho ERK, Nitrated ERK and ubiquitinated ERK. Results are representative of three independent experiments

in controls and increased extensively by treatment with SNP (nitrated ERK, lanes 1 and 4). SNP treated cells that were irradiated also showed considerable nitration of ERK (Fig. 1, nitrated ERK, lanes 5 and 6). ERK was found to be ubiquitinated appreciably with IR treatment (lane 2 and 3). SNP treatment also resulted in increased ubiquitination as compared to control. However when SNP treatment was followed by irradiation (lane 5 and 6), there was a time dependent decrease in ubiquitination, as compared to IR (lane 2 and 3) or SNP alone (lane 4).

Figure 2 shows the blots and representative histograms of tyrosine phosphorylation, nitration and ubiquitination of

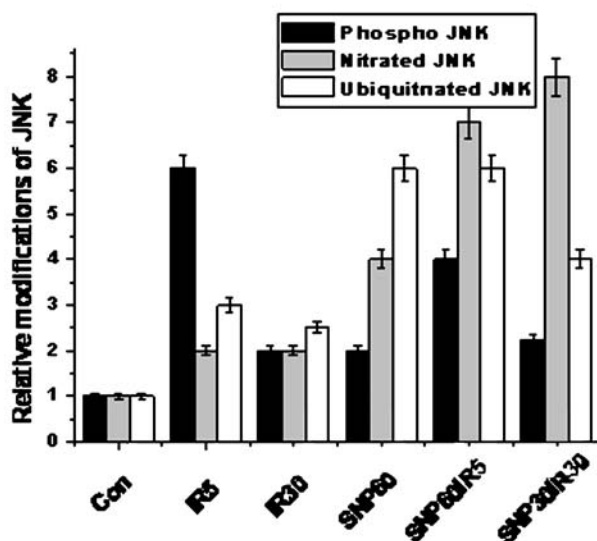
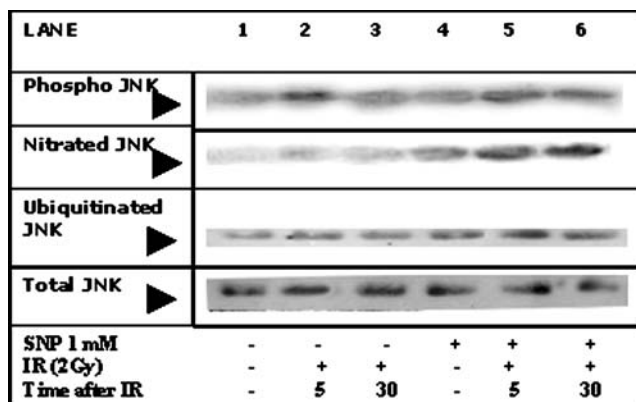


Fig. 2 Effect of SNP and/or IR treatment on tyrosine phosphorylation, tyrosine nitration and ubiquitination of JNK: PEC were irradiated (2 Gy) and lysed after 5 and 30 min in the absence or presence of SNP (1 mM, 60 min). The total JNK was immunoprecipitated from the lysates with anti rabbit anti JNK followed by immunoblotting. Membranes were then probed by anti mouse anti JNK, anti phospho-JNK, anti-nitrotyrosine or anti-ubiquitin. **a** One of three resulting hybridization images of each is shown here. **b** Histogram representing relative intensities of phospho JNK, Nitrated JNK and ubiquitinated JNK. Results are representative of three independent experiments

JNK after irradiation and SNP treatment. Like ERK, JNK also showed extensive phosphorylation at 5 min after irradiation and then a decline (lane 2 and 3). SNP treatment inhibited the irradiation induced increase in phosphorylation but extensively increased JNK's nitro tyrosine immunoreactivity which was not transient like its phosphorylation (Fig. 2, lane 5 and 6). Tyrosine nitration of JNK also increased in presence of SNP alone (lane 4). JNK was found to be ubiquitinated appreciably with SNP treatment (lane 4). However when SNP treatment was followed by irradiation, there was a time dependent decrease in ubiquitination, as compared to SNP alone (lane 5 and 6).

The total ERK and JNK as observed in immunoprecipitated lysates was unchanged with SNP treatment alone or followed by irradiation and has been used as a control for immunoprecipitation (Figs. 1 and 2).

Estimation of ERK and JNK activities

Figure 3 shows the phosphorylation of Elk and *c-jun* which are substrates for ERK and JNK respectively and hence are the indicators of their activities. A high basal phosphorylation of elk which decreased 5 min after irradiation and then came back to control levels after 30 min was observed (lanes 2 and 4, Fig. 3a). This did not conform to the phosphorylation status of ERK and was in fact inverse of that. Treatment with both SNP and IR also led to a decrease in phosphorylation of elk (lanes 5 and 6, Fig. 3a).

c-jun showed increased phosphorylation after irradiation which was persistent till 30 min unlike the activation of its precursor, JNK (Fig. 3a, lanes 2 and 4; Fig. 3c). The IR induced phosphorylation of *c-jun* was further enhanced by pretreatment with SNP for 30 min indicating that in spite of inhibition of phosphorylation of its precursor, the transcription factor *jun* could be activated (lanes 5 and 6).

Splenic lymphocytes MAPK phosphorylation after SNP/IR treatment

It was likely that the inhibition of phosphorylation by SNP observed in this study could be characteristic of macrophages since they are NO producing cells and are habituated to operating in an NO environment and that other cells may not respond to SNP in the same way. To test this hypothesis the splenic lymphocytes were treated in the same way i.e. treated with SNP prior to irradiation and the phosphorylation of the ERK was looked at in the lysate (Fig. 4). The response of the ERK was similar to the macrophages and was more prominent. ERK showed considerable inhibition of irradiation induced phosphorylation after SNP treatment (compare lanes 3 and 4, 6 and 7, 9 and 10) but unlike in the macrophages, does not recover its phosphorylation even after 180 min.

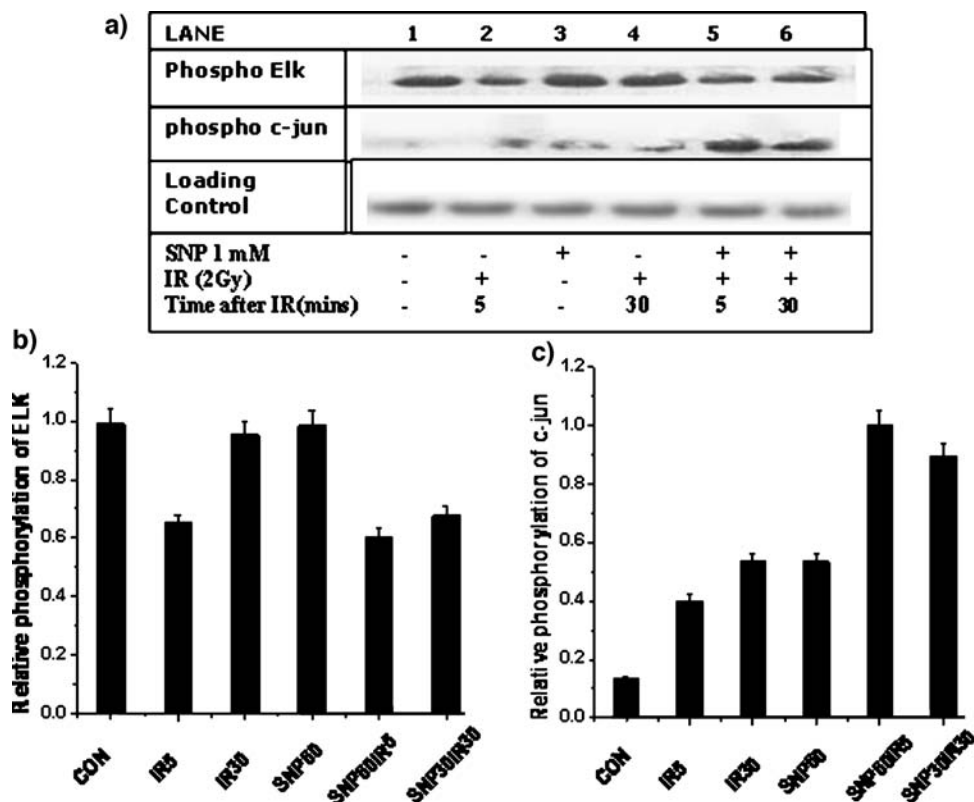


Fig. 3 Effect of SNP and/or IR treatment on phosphorylation of Elk and *c-jun*: PEC were irradiated (2 Gy) and lysed after 5 and 30 min in the absence or presence of SNP (1 mM) for 60 min. The cell lysates were immunoblotted and probed with anti phosphoElk or anti-phospho *c-jun*. **a** One of three resulting hybridization images of phosphoElk and phospho *c-jun* is shown here. Prominent band

obtained in Ponceau S staining of membrane shown here was taken as protein loading control and all other band intensities were divided by respective Ponceau band. **b** and **c** Histograms representing relative intensities of phospho Elk and phospho *c-jun* respectively. The lanes of histogram have been rearranged for comparison. Results are representative of three independent experiments

Discussion

MAPKs have been implicated in proliferation, differentiation (Das et al. 2000) and activation (Carter et al. 1999) of macrophages. MAPK pathways are known to get activated upon irradiation in many cells including macrophages (Dent et al 2003). In our earlier study we had observed that exposure of macrophages to SNP, a NO donor, led to a complete inhibition of the radiation induced phosphorylation of ERK and JNK. Despite the complete inhibition of MAPK phosphorylation; the viability, caspase 3 activity and phagocytic efficiency of macrophages were unaffected indicating that MAPK signaling was still operative (Narang et al. 2008). Since SNP is a nitric oxide donor, which in presence of radiation forms nitrating species, it is quite likely that the MAPKs are nitrated at tyrosines. In this study, the tyrosine nitration and ubiquitination of these MAPKs was looked at. The phosphorylation, nitration and ubiquitination of ERK and JNK were observed at 0, 5 and 30 min. Both ERK and JNK were activated with radiation dose of 2 Gy as early as 5 min and then showed a decline. But exposure to an NO donor led to inhibition of radiation induced phosphorylation of ERK and JNK (Figs. 1 and 2,

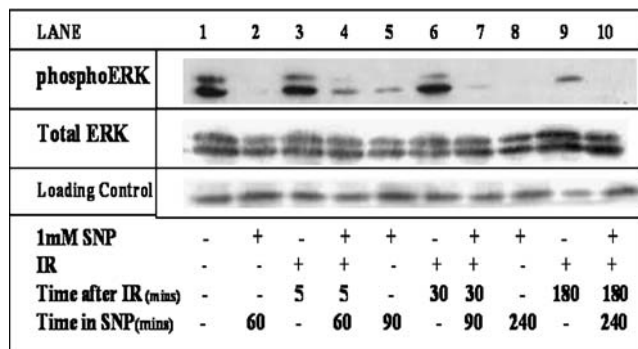


Fig. 4 Effect of SNP and/or irradiation with respect to time of treatment on phosphorylation status of ERK in splenic lymphocytes: 10⁶ cells/ml were treated with radiation dose of 2 Gy or with SNP (400 μM) and then lysed at indicated time periods (5, 30 and 180 min); lanes 3, 6 and 9. One set of cells was pretreated with SNP (0.4 mM) for 60 min before irradiation and then lysed after 5, 30 and 180 min; lanes 4, 7 and 10. SNP treatment alone was given for 60, 90 and 240 min; lane 2, 5 and 8. One of three resulting hybridization images of phospho-ERK and total ERK are shown here. Prominent band obtained in 1% Ponceau staining of the membrane is given as protein loading control

phospho ERK and JNK, compare lanes 2, 3 with 5, 6). Treatment with an NO donor led to increase in tyrosine nitration which further increased when NO treated macrophages were irradiated (Figs. 1 and 2, nitrated ERK and JNK, lanes 3–5). This could be because the reactive oxygen species generated following irradiation react with nitric oxide to generate reactive nitrogen species like peroxynitrite (Reiter et al. 2000) which are capable of nitrating the tyrosine residues in proteins. The nitration and phosphorylation did not seem to take place simultaneously in the kinases. At the time points when the nitration is extensive, phosphorylation is partially inhibited (Figs. 1 and 2, lanes 5 and 6).

Ubiquitination was looked for to check if they had been tagged for degradation. The results show that ubiquitination did not go hand in hand with nitration. Ubiquitination was in fact inhibited when macrophages were treated with both SNP and radiation as compared to treatment with SNP alone. However, it's been recognized that ubiquitination not only just tags the proteins for degradation but may play important role as a modification controlling various aspects of signaling like duration, magnitude or localization (Schnell and Hicke 2003; Di Fiore et al. 2003). MAPK pathways also have been shown to be under regulation by ubiquitination where MEKK, ubiquitinates its precursors including *c-jun* and ERK1c dictating their stability and localization respectively (Laine and Ronai 2005). That ubiquitination is not taking these nitrated proteins (ERK and JNK) for degradation is evident from the fact that following irradiation and SNP their total content did not change. However, since ubiquitination of these proteins is yet operative, it may play a role in regulation of MAPK signaling.

We further looked at the transcription factors acted upon by these kinases to see the translation of the radiation and nitric oxide stress on the effectors of these pathways. Though Elk and *c-jun* were initially known to be preferred targets of ERK and JNK respectively (Shaulin and Karin 2001; Davis 2000), the emerging studies of crosstalk between these pathways have blurred the specificity of substrate assigned to each. Many reports have shown the phosphorylation of elk by JNK (Zhang et al. 2007; Yang et al. 1998) and vice versa i.e. phosphorylation of *c-jun* by ERK (Leppa et al. 1998).

In our studies we looked at the phosphorylation of these downstream effectors, elk and *c-jun*, where *c-jun* was not phosphorylated to large extent after radiation or nitric oxide alone. But after both radiation and nitric oxide treatment, phosphorylation of *c-jun* increased significantly while elk showed decrease in phosphorylation. Previous studies have also shown that pro-survival ERK pathway and pro-apoptotic JNK pathway act in dynamic balance with respect to radiation exposure, with one pathway excluding the activation of other (Carter et al. 1998; Reardon et al. 1999) also sustained activation of JNK is known to block ERK

activation by mitogenic factors. In this study we have observed this shifting of phosphorylation from elk to *c-jun* between the two pathways with respect to dual stress (radiation and nitric oxide). This is logical since the two stresses need to be indicated and translated into effect in the cell via *c-jun* activation which is a stress responsive factor (Hibi et al. 1993; Derijard et al. 1994). Moreover, these transcription factors are not known to act in an independent manner but in association with other proteins (Karin et al. 1997). The oligomeric complex that forms by association of two or more transcription factors regulates different target genes and executes different biological functions depending upon its constituents.

After both the stresses at 60 min, when phosphorylation of JNK is inhibited and its nitration extensive, the phosphorylation of *c-jun* was significantly high (Fig. 3a, lanes 5 and 6). This indicates that nitrated JNK may be capable of transmitting a signal as effectively as its phosphorylated counterpart. Although both ERK and JNK were nitrated, only *c-jun* was phosphorylated, elk was not. This might indicate the specificity of regulation by nitration where it inhibits one kinase while activating the other.

Multiple modifications are a normal mode of signal transduction in physiology. Besides phosphorylation, nitration and nitrosylation of many signaling proteins has been shown to activate them, e.g. src (Macmillan-Crow et al. 2000) and many neurosignaling proteins (Jaffery et al. 2001). Tyrosine nitration has been shown to activate protein kinase C epsilon (Balafanova et al. 2002), cytochrome c (Cassina et al. 2000) and fibrinogen (Vadseth et al. 2004). Many crucial proteins are also known to undergo nitration with a change in their properties (Yamakura et al. 1998; Hellberg et al. 1998 and Macmillan-Crow et al. 2000) and also their biological half life. Nitrosylation of bcl₂ inhibits its ubiquitin mediated proteasomal degradation (Azad et al. 2006) while nitration of FLICE increases its proteasomal degradation (Chanvorachote et al. 2005).

In this study the nitration of JNK may extend its biological half life such that the signal is prolonged or is amplified which is reflected in the extensive phosphorylation of its substrate, *c-jun*. Many studies have established that time-course of activation of ERK and JNK plays important role in deciding the final outcome of the cell e.g. in the macrophages time course of ERK takes it either towards proliferation or differentiation (Valledor et al. 2000). Following radiation damage also it has been shown that transient activation of ERK led to proliferation whereas persistent activation led to cell cycle arrest followed by apoptosis (Deak et al. 1998; Poon et al. 1996). In Jurkat cells persistent but not transient activation of JNK led to apoptosis (Faris et al. 1998). That the life of these signaling proteins has been extended is evident by the fact that their ubiquitination and hence the ensuing degradation are inhibited.

In conclusion, MAPKs may be under regulation of different post translational modifications during initial stage of nitric oxide release during macrophage activation, three of which have been dealt with in this study. This interplay might affect time course of their activation. However, it is quite likely that other post translational modification, yet unknown, may also participate in signaling.

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