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# Effect of Nitric Oxide Donor and Gamma Irradiation on MAPK Signaling in Murine Peritoneal Macrophages

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**Abstract** Irradiation (IR) of cells is known to activate enzymes of mitogen activated protein kinase (MAPK) family. These are known to be involved in cellular response to stress and are determinants of cell death or survival. When radiotherapy is delivered to malignant cells, macrophages, being radioresistant, survive, get activated, and produce large amounts of nitric oxide. As a result of activation they recognize and phagocytose tumor and normal cell apoptotic bodies leading to tumor regression. In this study, the MAPK signaling in peritoneal macrophages was investigated which plays an important role in its various functions, in an environment which is predominantly nitric oxide, as is after IR. The behavior of macrophages in such an environment was also looked at. The three MAPK (ERK1/2, p38, and JNK) respond differently to Sodium nitroprusside (SNP) alone or IR alone. All the three were activated following IR but only JNK was activated following SNP treatment. Surprisingly, when both the stresses were given simultaneously or one after the other, this differential response was lost and there was a complete inhibition of phosphorylation of all the three MAPKs, irrespective of the order of the two insults (IR and SNP). The noteworthy observation was that despite the complete inhibition of MAPK signaling there was no effect on either the viability or the phagocytic efficiency of peritoneal macrophages. J. Cell. Biochem. 103: 576–587, 2008. 

**Key words:** phosphorylation; sodium nitroprusside; SNP; irradiation; mitogen activated protein kinase; MAPK; macrophages

Mitogen activated protein kinase (MAPK) signaling pathways translate extra cellular and intracellular stimuli into cellular responses. The MAP kinase family is composed of the ERK 1/2, p38, and SAPK/JNK pathways. Although distinct in their activation, there is considerable co-operation between these kinases and many substrates are shared between the pathways [Cobb, 1999]. This family of kinases is important for a wide spectrum of cell functions such as proliferation [Pages et al., 1993; Mansour et al., 1994], apoptosis [Cross et al., 2000; Kong et al., 2000], cytokine biosynthesis [Baldassare et al., 1999; Kovalovsky et al., 2000], and cytoskeletal reorganization [Landry and Hout, 1995; Guay et al., 1997]. All MAP kinases are highly conserved serine-threonine kinases that are activated by upstream MAPK kinases through a Thr-Xxx-Tyr phosphorylation motif [Martin-Blanco, 2000]. Studies have shown that, in general, the ERK 1/2 pathway is activated by growth factors, mitogenic stimuli, and tumor promoters [Cobb, 1999], the p38 and SAP/JNK pathways are activated by environmental stress and inflammatory cytokines [Zu et al., 1998; Bellmann et al., 2000; Kovalovsky et al., 2000].

In macrophages these pathways are activated upon binding of growth factors and inflammatory cytokines to specific receptors and are essential for the activation of cytokine gene transcription. The activated MAPK are responsible for phosphorylating and activating numerous transcription factors which stimulate the synthesis of various inflammatory proteins including the cytokines TNF-$\alpha$, IL-1$\beta$, and IL-6 and other important enzymes like cyclooxygenase-2 and iNOS [Chan et al., 1999; Chen et al., 1999]. Large amounts of nitric oxide, produced via iNOS activation in macrophages, shows cytostatic or cytotoxic activity against pathogens and tumor cells and also induces apoptosis in macrophages themselves [Albina et al., 1993; Messmer et al., 1995]. Nitric oxide is known to

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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 [Monteiro et al., 2000; Bernabe et al., 2001; Oliveira et al., 2003], other modifications like *S*-nitrosylation [Mondoro et al., 1997; Deora et al., 2000], nitration [Cassina et al., 2000; Balafanova et al., 2002; Vadseth et al., 2004], or by inactivation of phosphatases [Takakura et al., 1997].

When radiotherapy is delivered to malignant cells, macrophages being radioresistant survive, get activated by radiation and regulate inflammatory and immunological responses. They also recognize and phagocytose tumor and normal cell apoptotic bodies leading to tumor regression.

Most of the studies regarding macrophage viability and function after irradiation (IR) have been done at clinically irrelevant doses [Clarke and Wills, 1980; Sablonniere et al., 1983; Gallin et al., 1985; Lambert and Paulnock, 1987; Takahashi et al., 1990; Yifen et al., 1992] and also the information about MAPK signaling in primary macrophages is limited.

In the view of the above, it was therefore interesting to study the MAPK signaling and its modulation after IR under nitric oxide rich environment and how it effects the behavior of macrophages.

## MATERIALS 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 ($24 \pm 2^{\circ}$C) rooms with a 12-h 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 as 70% of these cells are macrophages. PEC were isolated from animals by washing out the peritoneal cavity. In brief, RPMI1640 (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 2,500 rpm for 6 min, resuspended in RPMI1640 medium at concentration of $10^6$ cells/ml.

### Treatment of PEC

In first set of experiments, for studying time periods of phosphorylation after IR and/or Sodium nitroprusside (SNP) treatment, PEC ($10^6$ cells/ml) were either irradiated at 2 Gy (dose rate 0.1 Gy/sec, Gamma cell 220, AECL, Canada) or were treated with 1 mM SNP, and then lysed after different time periods (5 min, 30 min, and 1 h) as shown in figures (Figs. 1–3, lane 1–7).

For combined treatments (SNP and IR) as shown in figures (Figs. 1–5), following groups were made: one set of cells was treated with SNP and IR simultaneously and lysed after 30 min (group A–gpA). The second set was divided into two where one set of cells was preincubated with SNP (1 mM) for 30 min and then irradiated at 2 Gy and lysed after 5 min (gpD), the second set of cells was lysed after 30 min (gpC). The sequence of two insults was interchanged where the cells were irradiated (2 Gy) 30 min prior to addition of SNP (1 mM). Cells were kept in SNP for 30 min and then lysed (gpB).

Lysates were then processed for immunoblotting.

### SDS–PAGE and Western Blot Analysis

After treatment, cells were centrifuged and lysed with 100 μl SDS sample buffer (10% glycerol, 2% SDS, 100 mM DTT, 0.1% bromophenol blue, and 50 mM Tris-HCl (pH-6.8)). The lysates were boiled for 10 min and the proteins separated by 10% 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 either of following antibodies, anti-phosphotyrosine, (Calbiochem CN Biosciences), anti-phosphoERK (Promega), anti-phospho p38MAPK (Promega),

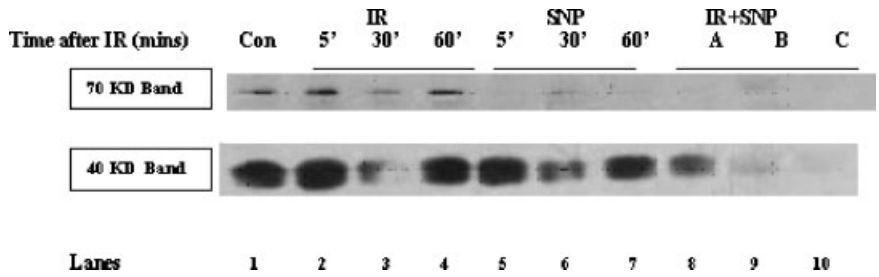


**Fig. 1.** Effect of SNP and/or irradiation with respect to time of treatment on phosphotyrosine levels in PEC: $10^6$ cells/ml were either treated with radiation dose of 2 Gy or with SNP (1 mM) and then lysed at indicated time periods (5, 30, and 60 min). **Lane 1**: untreated; **lanes 2–4**: PEC irradiated at 2 Gy and lysed after 5, 30, and 60 min, respectively; **lanes 5–7**: PEC treated with SNP for 5, 30, and 60 min, respectively. For combined treatment by IR and SNP; **lane 8**: cells were subjected to SNP treatment and irradiation together and lysed after 30 min (gpA); **lane 9**: 30 min after irradiation of PEC SNP (1 mM) was added and cells were again kept for 30 min before lysis, such that irradiation preceded SNP treatment of 30 min (gpB); **lane 10**: cells were preincubated with SNP (1 mM) and irradiated after 30 min and then lysed after 30 min such that SNP treatment preceded irradiation (gpC). After lysis the cells were subjected to SDS–PAGE followed by Western blotting using anti-phosphotyrosine antibody. One of three resulting hybridization images is shown here.

anti-activated JNK (Sigma), or anti-MKP-1 (Santacruz Biotech), followed by horseradish peroxidase (HRP) conjugated secondary antibody (Roche Molecular Biochemicals, Germany). The secondary antibody was detected using BM Chemiluminiscence Western Blotting Kit (Roche Molecular Bio chemicals, Germany). The band intensity was quantified by software Gelquant (version 2.7.0 DNR Bioimaging systems Ltd.). For protein control, the membranes were initially stained with Ponceau S (Sigma), the prominent band obtained in ponceau stain was taken as loading control and all the other band intensities were divided by respective lane's loading control intensity.

### Phagocytic Activity of PEC

Phagocytic activity of PEC treated with or without SNP and/or IR was estimated using FITC-labeled *E. coli*. For FITC-labeling, bacteria were grown at 37°C in a shaker incubator and collected at log phase of their growth. To the cell pellet, FITC dissolved in bicarbonate buffer, pH 10.0 was added (2.5 mg/mg bacterial protein) and the tube was kept on the rocker at room temperature for 2 h. The cells were washed three times with PBS to remove excess FITC. Cells were stored at 4°C in PBS and were used within 7 days. PEC were treated with SNP and/or IR as described previously, all treatment groups were washed with PBS and mixed with FITC labeled bacteria in 1:10 effector to target ratio. The mixture was centrifuged for 15 s to increase their interaction and were incubated at 37°C for 30 min. Ice-cold PBS was added to stop phagocytosis. Cells were visualized and counted under Carl-Ziess Axioscope microscope with high performance monochrome CCD camera. Total number (minimum 100 PEC per group) of macrophages in a given focus were observed under visible as well as blue illumination to distinguish macrophages with phagocytosed bacteria from macrophages without phagocytosed bacteria. Trypan blue (10 μl) was added to quench the fluorescence of surface bound and free floating bacteria (non-phagocytosed). Phagocytic efficiency of PEC in each treatment group was calculated as ratio of FITC positive PEC to that of total number of PEC.

### Effect of SNP and/or IR on Viability of PEC

Total number of viable PEC in each treatment group were enumerated by trypan blue dye exclusion method. PEC were treated with SNP and/or IR for 30 min or 1 h as described previously. After treatment, they were washed, resuspended in RPMI1640 with 2% FCS and incubated at 37°C for 4 h. Minimum 100 cells per group were counted and the data points show mean ± SEM from three independent experiments.

### Caspase 3 Activity

Caspase 3 activity was assayed in PEC to see the induction of apoptosis in different treatment groups. $10^6$ PEC/ml in each group were treated with SNP and/or IR for 30 min or 1 h as described earlier. After treatment, they were washed, resuspended in RPMI1640 with 2% FCS and incubated at 37°C for 4 h. Caspase 3

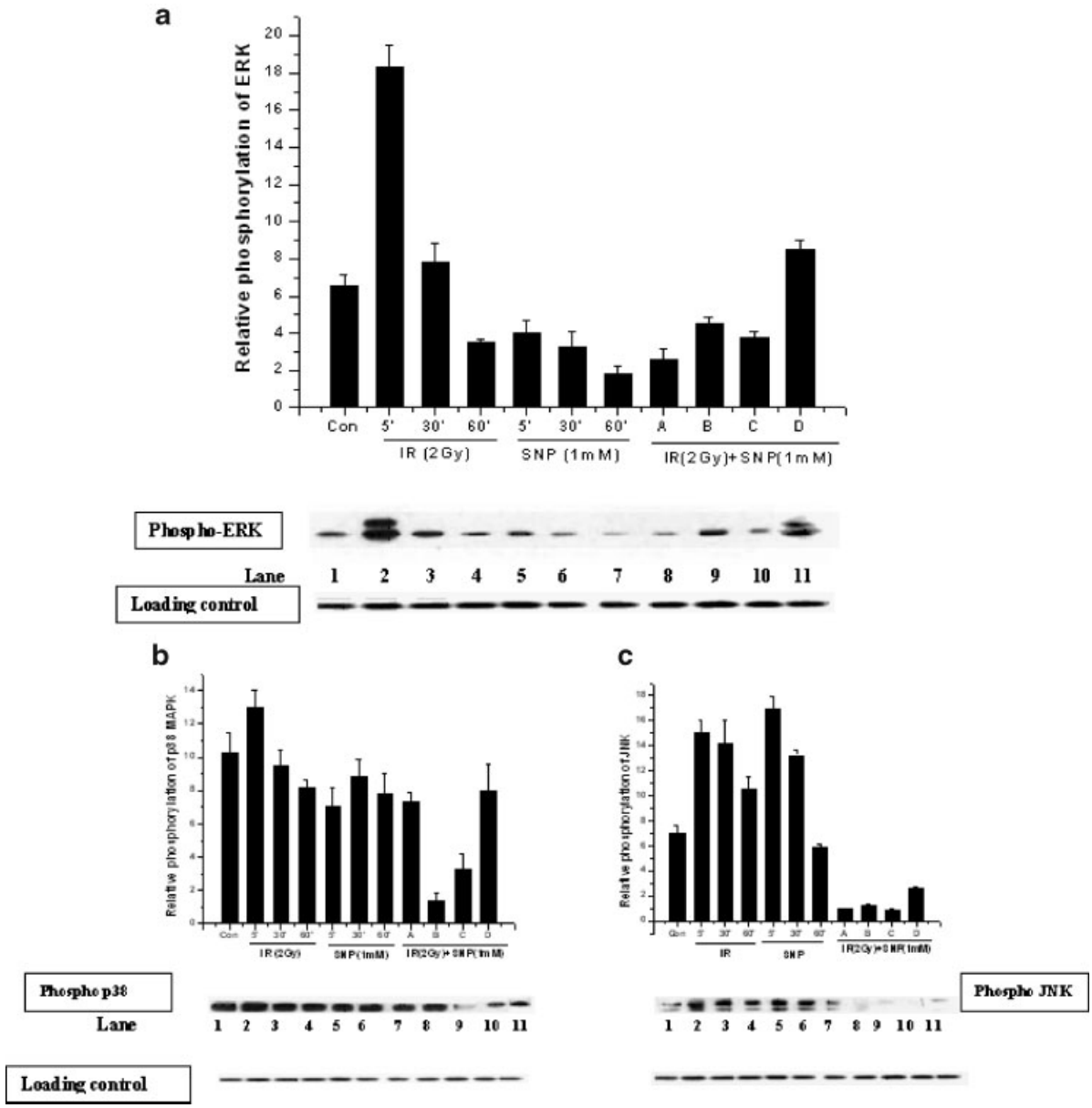


**Fig. 2.** Effect of SNP and/or IR treatment on tyrosine phosphorylation of three MAPKs, ERK1/2, p38 MAPK and JNK. $10^6$ cells/ml were either irradiated at a dose of 2 Gy (**lanes 2–4**) or incubated with SNP (1 mM) (**lanes 5–7**), then lysed at different time periods (5, 30, and 60 min). For dual treatments as described in Materials and Methods, one set of cells was treated with SNP and irradiation simultaneously, and then lysed after 30 min (**lane 8**, gpA). The second set of the cells was irradiated, after 30 min SNP was added and cells lysed 30 min after such that irradiation preceded SNP treatment (**lane 9**, gpB). The third set was incubated with SNP for 30 min, irradiated, reincubated for 30 min and than lysed such that SNP treatment preceded irradiation (**lane 10**, gpC). The fourth set of the cells was incubated with SNP for 30 min, irradiated and lysed after 5 min (**lane11**, gpD). Lysates from different treatment groups were subjected to SDS–PAGE followed by Western blotting using (**a**) anti-phosphoERK antibody (**b**) Anti-phospho p38 MAPK, and (**c**) anti-JNK. One of three resulting hybridization images is shown here. The intensity of bands obtained was plotted versus time after irradiation/SNP; data points represent means from three separate experiments.

activity was estimated in lysate using caspase 3-activity assay kit (Sigma). Briefly, PEC were lysed in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, and 5 mM DTT) for 15–20 min at 4°C and then centrifuged (16,000*g* for 15 min, 4°C). Lysate (10 μl) was incubated with 10 μl of substrate (Ac-DEVD-pNA) in assay buffer (20 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA). After overnight incubation at 37°C, the colorimetric detection of cleaved pNA product was estimated at 405 nm using ELISA reader (Bio-tek Instruments, Inc.). The

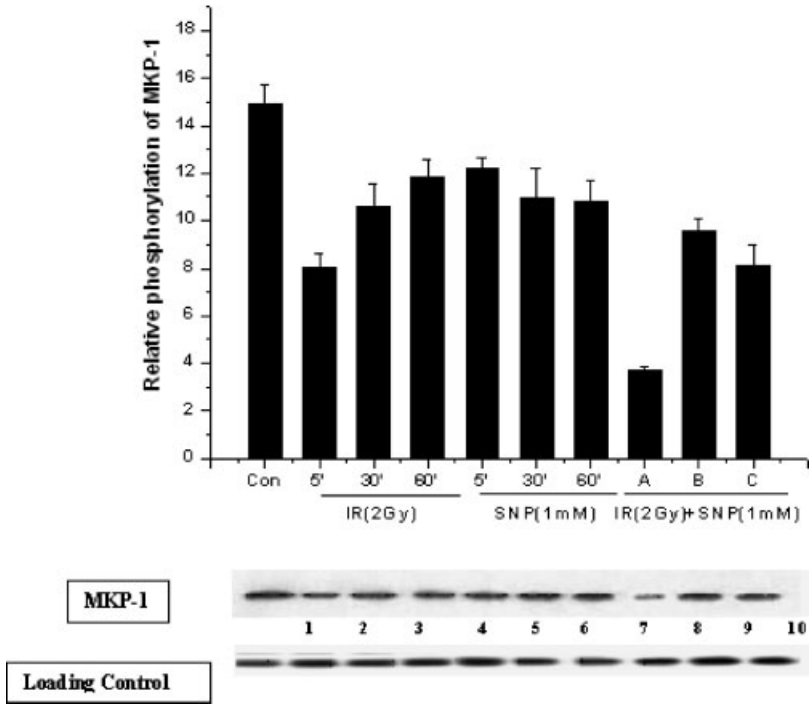


**Fig. 3.** Western blot of MKP-1 after treatment with SNP and/or irradiation. $10^6$ cells/ml were either irradiated at a dose of 2 Gy (**lanes 2–4**) or incubated with SNP (1 mM) (**lanes 5–7**), lysed at different time periods (5, 30, and 60 min). For dual treatments, one set of cells was treated with SNP and irradiation simultaneously, was kept for 30 min and then lysed (**lane 8**, gpA). The second set of the cells was irradiated, kept for 30 min, SNP was then added and cells were lysed after 30 min incubation with SNP such that irradiation preceded SNP treatment (**lane 9**, gpB). The third set was incubated with SNP for 30 min, irradiated, reincubated for 30 min and than lysed such that SNP treatment preceded irradiation (**lane 10**, gpC). After lysis the cells were subjected to SDS–PAGE followed by Western blotting using anti-MKP-1 antibody. One of three resulting hybridization images is shown here. The intensity of bands obtained was plotted versus time after irradiation/SNP; data points represent means from three separate experiments.

data points show mean ± SEM from three independent experiments.

## RESULTS

### Effect of SNP and/or Irradiation on Total Tyrosine Phosphorylation in PEC

Total phosphotyrosine levels in PEC were looked at after IR (2 Gy) alone or with SNP (1 mM) alone for varied time periods (5′, 30′, and 60′) (Fig. 1, lane 1–7). This resulted in two prominent bands, with apparent molecular weights of 40 and 70 kDa. In both the bands after IR substantial increase in phosphorylation at 5 min, a drop at 30 min, and again increase at 1 h was observed. Treatment with SNP resulted in complete abolishment of phosphorylation of the 70 kDa band while 40 kDa band showed similar pattern of activation as was seen with IR.

The sequence of the two stresses (SNP and IR) was changed to see how each stress effects the phosphorylation of the other. For this SNP was added either at the time of IR (Fig. 1; lane 8, gpA), or 30 min after IR (lane 9, gpB) or 30 min before IR (lane 10, gpC). The cells were lysed 30 min after the last treatment. Under all the three conditions, phosphorylation was totally absent that is irrespective of the sequence of the two stresses (SNP and IR) phosphorylation was totally inhibited when compared to control. Since MAPK activation via phosphorylation is central to normal functioning of any cell, the strong inhibition of basal and IR induced tyrosine phosphorylation by SNP led us to investigate effect of SNP on phosphorylation of MAPKs in macrophages which are known to get activated by reactive oxygen/nitrogen species and IR.

### Phosphorylation Pattern of MAPKs After Irradiation, SNP Treatment, and Both Together

With IR alone, robust activation of ERK was seen at 5 min after IR which reduced with time and came down to control levels at 60 min (Fig. 2a, lane 1–4). No increase in phosphorylation after treatment with SNP was observed;

rather a time dependent decrease in ERK phosphorylation with SNP (lane 5–7) which was much below control levels even 1 h after SNP treatment was seen. To study the effect of SNP on IR induced phosphorylation of ERK and to see the effect of presence of SNP before or after IR, we followed its phosphorylation under conditions as described in Materials And Methods where the cells were either treated with SNP and IR simultaneously (lane 8, gpA) or IR preceded SNP treatment (lane 9, gpB) or SNP treatment preceded IR (lane 10, 11; gpC, gpD). IR induced activation of ERK observed 5 min after IR was inhibited when cells were preincubated with SNP for 30 min (compare lane 2 with 11). When the cells were treated with SNP and IR simultaneously the phosphorylation of ERK at 30 min (lane 8, gpA) was significantly reduced as compared to when the cells that were treated with either of the stresses alone for 30 min (lane 3,6).

Like ERK, the phosphorylation of p38 Kinase (Fig. 2b) showed similar pattern with IR with an increase in phosphorylation at 5 min and return to control levels with time (Fig. 3, lanes 2–4). With SNP alone, no increase in phosphorylation was observed, in fact it was even lesser than the control (lanes 5–7). The IR induced increase in phosphorylation observed at 5 min was also inhibited when cells were preincubated with SNP (compare lane 2 and 11). The phosphorylation of p38 was much less than control for gpC (lane 10) and gpB cells (lane 9), though the simultaneous treatment with SNP and IR did not lead to such a drastic inhibition of phosphorylation (gpA, lane 8).

The third kinase JNK, the stress induced kinase (Fig. 2c), also showed an increased phosphorylation at all the time periods after IR and remained higher than the control levels till 60 min (lanes 2–4). Unlike the other MAPKs, JNK showed an increase in phosphorylation after treatment with SNP. Percentage increase in phosphorylation was equal to that observed with IR alone and was maximal at 5 min and then decreased with time reaching control levels at 60 min (lanes 5–7). However, when the two stresses were given either simultaneously or one after the other, it led to complete abolishment of phosphorylation of JNK irrespective of the order of the stresses in which they were given (lanes 8–11). Like the other two kinases, ERK and p38, prior addition of SNP completely abolishes the IR induced increase in phosphorylation achieved after 5 min of IR (Fig. 2c, compare lane 2 and 11).

The phosphatases are said to play an active part in the maintenance of the phosphorylated state. The expression of MKP-1 (Fig. 3), which is a phosphatase for MAPK family of enzymes, especially ERK was looked at. MKP-1 expression was found to be below control levels at all time periods after IR or SNP treatment. At 5 min after IR there was a sharp decrease in MKP-1 expression which was the time point of maximum phosphorylation of all the three MAPKs. But in the cells treated with SNP and IR simultaneously (gpA), there was again a very significant decrease in MKP expression (lane 8) which was not concurrent with any increase in phosphorylation of the any of MAPKs.

### Effect of SNP and/or IR Phagocytic Efficiency of PEC

Phagocytic efficiency of macrophages was tested as per protocol in Materials and Methods to see the effect of SNP and/or IR on PEC phagocytic efficiency. No change in phagocytic efficiency of the cells with either or both the treatment was observed. Macrophages which were irradiated 30 min prior to SNP and were kept for another 30 min before testing did show 1.3-fold increase in their phagocytic efficiency (Fig. 4a,b).

Macrophages which were treated with SNP 30 min prior to IR showed appreciable increase in apoptosis that is 25% which was five times more than observed with control.

### Effect of SNP and/or IR on Viability and Initiation of Apoptosis of PEC

The MAPK phosphorylation and activation is of paramount importance to the cell since the message of the incoming stress has to be translated into death or survival. The complete inhibition of phosphorylation of MAPKs after treatment with both SNP and IR therefore led us to investigate their effect on macrophage viability and caspase 3 activity. Viability was found to be 85% with all the treatment groups (Fig. 5a).

Caspase 3 activity was then looked at to see if cells have initiated the process of apoptosis (Fig. 5b). Although the cells treated with SNP and IR alone or simultaneously did not show any increase in apoptosis as compared to control, the cells which were treated with SNP 30 min after IR showed a twofold increase in

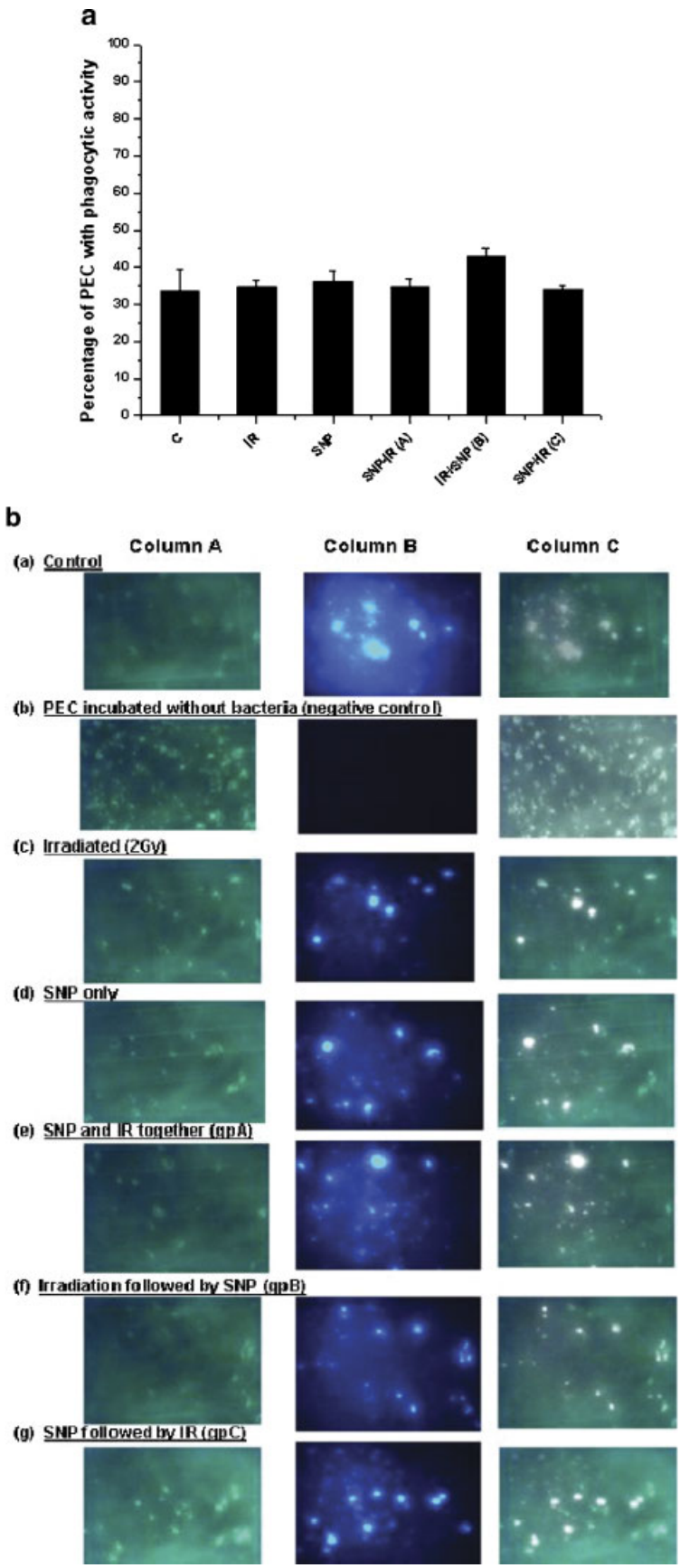
a
Percentage of PEC with phagocytic activity
100
90
80
70
60
50
40
30
20
10
0
C
IR
SNP
SNP+IR (A)
IR+SNP (B)
SNP+IR (C)
b
Column A
Column B
Column C
(a) Control
(b) PEC incubated without bacteria (negative control)
(c) Irradiated (2Gy)
(d) SNP only
(e) SNP and IR together (gpA)
(f) Irradiation followed by SNP (gpB)
(g) SNP followed by IR (gpC)

Fig. 4.

caspase 3 activity. Cells which were treated with SNP 30 min prior to IR showed fivefold increase in caspase activation that is 25% of the cells showed caspase activity as against 5% of the control cells.

## DISCUSSION

In present study the effect of IR on MAPK signaling in presence or absence of SNP has been looked at in the macrophages. The total tyrosine phosphorylation and activation of MAPKs was looked at from 5 to 60 min. This was done to obtain the whole activation pattern of kinases with time because the duration of phosphorylation is considered to be as important as the extent of phosphorylation in determining the outcome of any stimuli. This has been demonstrated in primary culture of rat hepatocytes where acute or chronic activation of MAPK cascade determined whether DNA synthesis was stimulated or inhibited [Tombes et al., 1998] and in jurkat cells where persistent but not transient activation of JNK led to apoptosis [Faris et al., 1998]. 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 [Poon et al., 1996; Deak et al., 1998].

In our studies the activation pattern of total tyrosine phosphorylation in PEC after IR alone, SNP alone and IR in presence of SNP followed a biphasic response as has been shown in previously by Singh et al. [2005] in BCR signaling in lymphocytes. This is because initial phosphorylation may be a stress response which sets in very early and is expected to die off once the stimulant/stress is removed and may express as another wave of response where the same proteins may or may not be involved. Treatment with SNP alone resulted in significant increase in phosphorylation of some protein while totally inhibiting the other, indicating the specificity of SNP towards inducing or inhibiting phosphorylation. The ROS generated following IR react with nitric oxide to generate reactive nitrogen species [Reiter et al., 2000]. The decrease in tyrosine phosphorylation in cells irradiated 30 min after SNP treatment could be due to nitration of the tyrosine moieties [Pfieffer et al., 2000; Thomas et al., 2002]. Reiter et al. [2000] have shown that some proteins undergo tyrosine nitration when cells were exposed to NO and $O_2^-$ simultaneously, but not when exposed to NO or $O_2^-$ alone. However, our results showed a substantial inhibition of phosphorylation in irradiated cells exposed to SNP 30 min after IR when only secondary radicals are present (treatment gpB, or lane 9 in Fig. 2a–c). Hence, the sequence of exposure to IR and SNP did not make a difference in affecting tyrosine phosphorylation.

Having found such significant variation in phosphorylation after SNP treatment and IR, it was of interest to examine whether the three crucial MAP kinases and their behavior following SNP treatment and IR in PEC was also altered. Our present results showing activation of ERK, p38, and JNK after IR (Fig. 2a–c, lane 2–4) are in agreement with previous reports [Dent et al., 2003a,b].

The high amount of phosphorylation of all the three MAPKs at 5 min after IR correlated with the significantly reduced expression of MKP-1, a MAPK specific phosphatase, at that time point.

Contrary to the previous reports where nitric oxide and peroxynitrite [Klotz et al., 1997; Bernabe et al., 2001; Oliveira et al., 2003] have been shown to activate ERK1/2, p38 MAP kinase, and JNK, we did not observe any activation of ERK1/2 or p38 kinase, in fact the phosphorylation was even lesser than the control levels in SNP treated cells. JNK, which is a stress activated protein kinase, did show increased phosphorylation with SNP. MAPKs, hence showed a differential response to nitric

**Fig. 4.** Effect of SNP and/or IR on phagocytic efficiency of PEC: (**a**) The bars represent percent phagocytic activity in each treatment group. $10^6$ PEC/ ml in each group were treated with SNP and/or irradiation for 30 min or 1 h as described in Materials and Methods. Treated or untreated PEC were mixed with FITC labeled bacteria in 1:10 effector to target ratio and incubated at 37°C for 30 min. Trypan blue (10 μl) was added to quench the fluorescence of surface bound and free bacteria (nonphagocytosed). Phagocytic efficiency of PEC in each treatment group was calculated as ratio of FITC positive PEC to that of total number of PEC. Minimum 100 cells per group were counted and the data points show mean ± SEM from three independent experiments. **b**: Images under fluorescent microscope depicting phagocytic activity of PEC, using FITC-labeled *E. coli*. Column A shows the image of PEC in a given focus under visible light, column B shows the image in the same focus under blue illumination to capture the fluoroscence of PEC with engulfed FITC labeled *E. coli* and Column C shows the overlay of two images to identify the PEC with *E. coli*. One representative image from all treatment groups is shown here. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

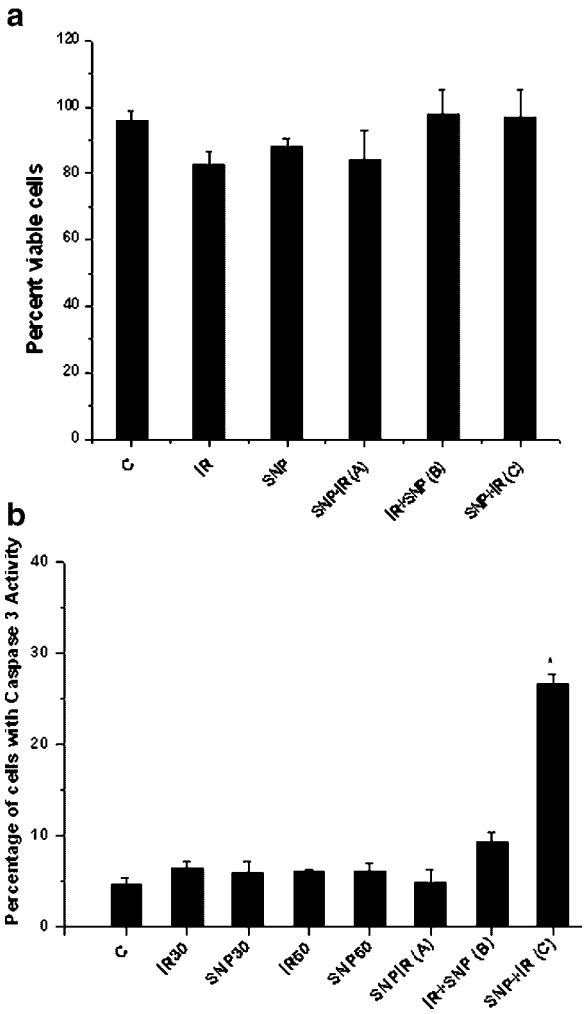


**Fig. 5.** Effect of SNP and/or IR on viability and caspase3 activity of PEC: (**a**) the bars represent the percentage of viable cells after SNP and/or IR treatment. Total number of viable PEC in each treatment group were enumerated by trypan blue dye exclusion. PEC were treated with SNP and/or irradiation for 30 min or 1 h as described in Materials and Methods. After treatment, they were washed, resuspended in RPMI1640 with 2% FCS and incubated at 37°C for 4 h and were then counted. Minimum 100 cells per group were counted and the data points show mean ± SEM from three replicates. Two such independent experiments were carried out. **b**: The bars represent the percentage of cells with caspase 3 activity. $10^6$ PEC/ ml in each group were treated with SNP and/or irradiation for 30 min or 1 h as described in Materials and Methods. After treatment, they were washed, resuspended in RPMI1640 with 2% FCS and incubated at 37°C for 4 h. The cells were then lysed and caspase activity was estimated in lysate. The data points show mean ± SEM from three independent experiments. $^*P < 0.01$, as compared to control.

oxide insult. The results of this study are supported by other observations where the effect of nitric oxide or peroxynitrite on members of src family of kinases was shown to be inhibited by higher concentrations of peroxynitrite [Di Stasi et al., 1999; Mallozzi et al., 1999]. JNK, which is involved mainly in pro-apoptotic signaling has been known to be activated by multiple stresses and is therefore often called stress activated protein kinase. Previous studies have shown that pro-survival ERK pathway and pro-apoptotic JNK pathway possibly act in dynamic balance with respect to radiation exposure, with ERK pathway acting to inhibit JNK pathway [Carter et al., 1998; Reardon et al., 1999; Vrana et al., 1999]. In this study the activation of JNK alone and not all the other two kinases will prepare the cell for incoming stress. This is logical since the cell has to respond under high concentration of nitric oxide.

The most intriguing finding of this study was that the treatment of cells both with SNP and IR resulted in substantial decrease in the phosphorylation or complete abolishment of MAPK phosphorylation irrespective of the order of the two insults. Although there was differential response of all the three MAPK to SNP alone or IR alone, when both the stresses were given this differential response was lost and all were completely inhibited.

Cellular phosphotyrosine levels are regulated by both kinases and phosphatases, and the duration of phosphorylated state of the kinases is very crucial in signaling. Studies in yeast and xenopus oocytes have shown that expression of phosphatases for MAPKs is transcriptionally induced by the same stimuli that activates its substrate, MAPK [Alessi et al., 1993; Doi et al., 1994]. The expression of MKP-1, a phosphatase for MAPK, was next looked at to see whether the strong inhibition of phosphorylation of MAPK is also reflected in its expression level. In our studies the time of reduced expression of MKP-1 coincided with the time at which the maximum phosphorylation of MAPKs was observed that is 5 min. The reduced expression of MKP-1 could be acting synergistically with reversible enzymatic inhibition of MKP-1 to keep kinases in an active form, resulting in amplified response. This decreased MKP-1 expression could be due to its rapid degradation after IR. The rapid degradation of phosphatases by calpain mediated pathway after UV radiation has been previously reported by Gulati et al. [2004]. Ionizing radiation has also been known to cause rapid degradation of some phosphatases like cdc25A phosphatase by proteasome pathway [Falck et al., 2001]. The drastic inhibition of phosphorylation after combined treatment with

IR and SNP though was not reflected in the expression of MKP-1.

Although macrophages are radioresistant, there is a possibility that with such a drastic inhibition of MAPK and tyrosine phosphorylation following treatment with both IR and SNP, the crucial functions of the cells and survival could be affected. No significant difference in the viability of macrophages after treatment with IR and/or SNP was observed. MAPK activation has been shown to play an important role in caspase 3 activation [Mohr et al., 1998; Callsen and Brune, 1999]. Caspase 3 activity was higher in the macrophages treated with SNP 30 min prior to IR or vice versa (gpB and C, Fig. 5b) where drastic inhibition of MAPK was observed. Our results showing 30–35% phagocytic efficiency in untreated control PEC are consistent with the results shown by Sharma et al. [2007] (Fig. 4a,b). The observed increase in phagocytic efficiency in treatment gpB could be due to longer incubation (60 min) after IR. Tyrosine phosphorylation and MAPK signaling have been shown to be crucial for cellular functions. However, our results show that inhibition of tyrosine phosphorylation and MAPK signaling by combined treatment with SNP and IR did not affect the viability and phagocytic function of macrophages (Figs. 4 and 5). This indicates that either macrophages are equipped with other pathways which can perform its essential functions or the complete abolishment of tyrosine phosphorylation may not lead to inactivation of MAPKs. Modification like tyrosine nitration of kinases 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]. We propose that under dual stress of radiation and SNP similar modification of these kinases may be responsible for their activation. However, this assumption needs to be experimentally verified.

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