

# Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: attenuation by selenite supplementation

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**Abstract** Peroxynitrite is a mediator of toxicity in pathological processes *in vivo* and causes damage by oxidation and nitration reactions. Here, we report a differential induction of mitogen-activated protein kinases (MAPKs) in WB-F344 rat liver epithelial cells by peroxynitrite. For the exposure of cultured cells with peroxynitrite, we employed a newly developed infusion method. At 6.5  $\mu\text{M}$  steady-state concentration, the activation of p38 MAPK was immediate, while JNK1/2 and ERK1/2 were activated 60 min and 15 min subsequent to 3 min of exposure to peroxynitrite, respectively. Protein-bound 3-nitrotyrosine was detected. When cells were grown in a medium supplemented with sodium selenite (1  $\mu\text{M}$ ) for 48 h, complete protection was afforded against the activation of p38 and against nitration of tyrosine residues. These data suggest a new role for peroxynitrite in activating signal transduction pathways capable of modulating gene expression. Further, the abolition of the effects of peroxynitrite by selenite supplementation suggests a protective role of selenium-containing proteins.

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**Key words:** Peroxynitrite; Signal transduction; p38; JNK1/2; ERK1/2; Selenoprotein

## 1. Introduction

Peroxynitrite ( $\text{ONOO}^-$ ), an oxidant produced *in vivo* by activated phagocytes and endothelial cells [1,2], is involved in tissue damage in a number of pathological conditions such as inflammation, ischemia-reperfusion and neurodegenerative disorders in humans and experimental animals (for review, see [3]). In response to mitogenic stimuli and environmental stresses, the mitogen-activated protein kinase (MAPK) pathways are activated leading to modulation of gene expression (for review see [4]). Activation of MAPKs is known to occur upon exposure to reactive species such as nitric oxide [5], superoxide [6], hydroperoxides [7,8] and UV irradiation [9–11]. The purpose of the present work was to examine whether peroxynitrite is able to activate MAPK signaling pathways.

Biological systems exhibit protective mechanisms to prevent damaging effects of peroxynitrite. Selenoproteins such as glutathione peroxidase (GPx) [12] or selenoprotein P [13] protect against peroxynitrite-mediated oxidation and nitration reactions. Selenite supplementation to cultured cells leads to an increased synthesis of selenoproteins, e.g. GPx [14]. Since selenite itself does not interact with peroxynitrite [15], we used selenite supplementation to cultured cells to investigate the

effects of selenoproteins on peroxynitrite-induced MAPK activation and nitration of protein-bound tyrosine residues.

## 2. Materials and methods

### 2.1. Cell culture

The WB-F344 rat liver epithelial cells were a kind gift from Dr. I.A. Cotgreave and Dr. L. Wärngård, Institute of Environmental Medicine, Karolinska Institute (Stockholm, Sweden). The cells were cultured in 30 mm dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, L-glutamine (2 mM), streptomycin (0.02 g/l), penicillin (20 000 IU/l). Cytotoxicity of sodium selenite was determined via the reduction of MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) to the corresponding formazan. Sodium selenite was supplemented to culture medium 48 h before exposure to peroxynitrite. Cells were grown to confluence and then serum-starved for 24 h before exposure to peroxynitrite to avoid a high background activation of ERK (MAPK) by growth factors present in serum.

### 2.2. Synthesis of peroxynitrite

Peroxynitrite was synthesized from sodium nitrite and  $\text{H}_2\text{O}_2$  using a quenched-flow reactor as described in [16]; excess  $\text{H}_2\text{O}_2$  was eliminated by the passage of the peroxynitrite solution over  $\text{MnO}_2$  powder. Peroxynitrite concentration was determined spectrophotometrically at 302 nm ( $\epsilon = 1705 \text{ M}^{-1} \text{ cm}^{-1}$ ).

### 2.3. Steady-state exposure of cultured cells to peroxynitrite

The cells were washed twice with PBS and the dishes were inserted upside-down into a cylinder containing 750 ml PBS, pH 7.2, maintained at 25°C. Peroxynitrite (120 mM) was infused (1 ml/min) with a micropump into the cylinder with the tube approximately 5 mm below the dish under constant stirring, resulting in a calculated steady-state concentration of 6.5  $\mu\text{M}$ . The steady-state input concentration of peroxynitrite was calculated by using the infusion rate of peroxynitrite (2.67  $\mu\text{M/s}$ ) and its decay rate in phosphate buffer at 25°C, pH 7.4 (0.41  $\text{s}^{-1}$ ) [17]. For lower concentrations, peroxynitrite was diluted in 0.1% (w/v) NaOH. Exposure of cells was for 3 min. The pH of the PBS did not change detectably when peroxynitrite was infused to generate a 1.6  $\mu\text{M}$  or lower steady-state concentration. Infusion of peroxynitrite to maintain a steady-state concentration of 6.5  $\mu\text{M}$  led to an increase in pH to 7.7. For '0  $\mu\text{M}$ ' peroxynitrite, predecomposed compound was employed. Peroxynitrite was decomposed by the addition of HCl; basic conditions were reconstituted by the addition of a similar amount of NaOH.

### 2.4. Phosphorylation of MAPKs

After exposure to peroxynitrite, cells were incubated on 30 mm dishes in serum free medium for the indicated time and then washed with PBS and lysed by scraping in 2×SDS-PAGE lysis buffer (125 mM Tris, 4% (w/v) SDS, 20% (v/v) glycerol, 100 mM DTT, 0.2% (w/v) bromophenol blue, pH 6.8). The lysates were heated at 95°C for 5 min and used for SDS-PAGE or frozen until use. Samples (15  $\mu\text{l}$ ) were subjected to gel electrophoresis on 12% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (ECL nitrocellulose, Amersham). Immunodetection of phosphorylated p38, JNK1/2 and ERK1/2 were with polyclonal anti-phospho-p38 (New England Biolabs, Schwalbach, Germany), polyclonal anti-active-JNK and polyclonal anti-active-MAPK (Promega, Mannheim, Germany) antibodies, respectively. Incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (Cappel/ICN,

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Eschwege, Germany) was followed by chemiluminescence detection (LumiGlo, New England Biolabs). After stripping, the membrane was reprobed with anti-total-p38 (New England Biolabs) or anti-total-JNK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies which served as gel loading and protein controls.

### 2.5. Assay of protein-bound 3-nitrotyrosine

After the separation of proteins by SDS-PAGE, Western blots using mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) were performed essentially as described in [12]. After the usual processing and incubation with a secondary goat anti-mouse antibody coupled to horseradish peroxidase (Cappel/ICN, Eschwege, Germany) chemiluminescence was detected (LumiGlo, New England Biolabs).

### 2.6. GSH peroxidase assay

GPx activity was followed spectrophotometrically at 340 nm as described in [18] with minor modifications. GPx activity was calculated from the rate of NADPH oxidation and expressed per mg protein. Protein was determined using the Bradford assay (Bio-Rad, Munich, Germany).

## 3. Results and discussion

### 3.1. Activation of MAP kinases by peroxynitrite

Due to the short half-life of peroxynitrite under physiological conditions ( $\sim 1$  s at pH 7.4, 37°C) [3], it is difficult to compare its effects on cultured cells *in vitro*, when peroxynitrite is added by bolus addition, with pathological conditions *in vivo*, where it is continuously formed. To mimic *in vivo* conditions, we developed a new infusion system (see Section 2) for exposure of cultured cells to a steady-state concentration of peroxynitrite. Exposure of serum-starved rat liver epithelial cells to peroxynitrite to a steady-state concentration of 6.5  $\mu$ M for 3 min resulted in a transient dual phosphorylation of p38 MAPK, JNKs and ERKs (Fig. 1). Activation of p38

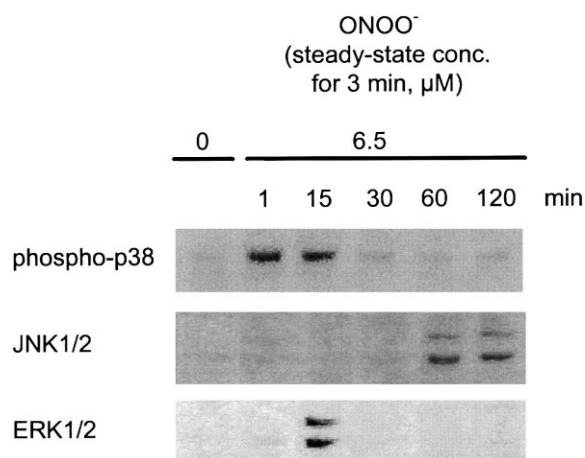


Fig. 1. Activation of MAP kinases upon exposure of rat liver epithelial cells to peroxynitrite. Serum-starved WB-F344 rat liver epithelial cells were exposed to peroxynitrite to maintain a steady-state concentration of 6.5  $\mu$ M for 3 min. For 0  $\mu$ M peroxynitrite, predecomposed compound was employed. After exposure, the cells were lysed immediately ( $\sim 1$  min) or incubated with serum-free medium at 37°C and lysed after 15, 30, 60 and 120 min. Activation was assessed as the dual phosphorylation of p38, JNK1/2 and ERK 1/2 in Western blots employing polyclonal phospho-specific antibodies. Equal protein loading was ascertained by stripping and reprobing with anti-total-p38 antibodies and anti-total-JNK antibodies (data not shown). The results of one of three independent experiments are shown.

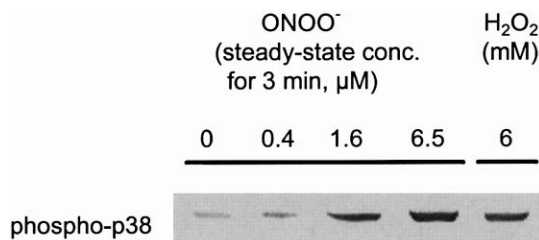


Fig. 2. Concentration dependence of p38 MAPK activation elicited by peroxynitrite. Serum-starved WB rat liver epithelial cells were exposed to different steady-state concentrations of peroxynitrite. Cells were lysed immediately after exposure and p38 phosphorylation was assessed as in Fig. 1. As a positive control, cells were treated with H<sub>2</sub>O<sub>2</sub> for 2 h in serum free medium.

and ERK1/2 was rapid and maximal immediately or 15 min after exposure to peroxynitrite, respectively. In contrast, JNK1/2 were activated only at 1 h after exposure. Activation of MAPK by peroxynitrite occurred in a concentration dependent manner, as exemplified by p38 phosphorylation (Fig. 2). Predecomposed peroxynitrite was infused to test whether the slight increase in pH to 7.7 did affect the activation of MAPKs (indicated as '0  $\mu$ M' in Figs. 1–3); MAP kinases were not activated under these conditions (Figs. 1–3).

This differential activation pattern of MAPKs by peroxynitrite is distinct from that reported for other stimuli. Nitric oxide-induced activation of p38 and JNK was maximal after a few minutes of treatment of Jurkat T cells with NO, whereas ERK1/2 activity rose slowly with the time of exposure to NO [5]. Superoxide exposure resulted in activated ERKs after 10 min in vascular smooth muscle cells [6]. Further, UVA or singlet oxygen show an activation of p38 and JNK but not ERKs in skin fibroblasts [9,19]. UVB leads to a more prolonged activation of p38, JNKs and ERKs, which remained activated for 8 h and 24 h, respectively [10]. UVC caused an increased activation of all three MAP kinases 15–30 min after irradiation [11,20,21]. Hydrogen peroxide-induced activation of MAPKs is different in that it shows a delayed and more prolonged response of p38 and ERK than that caused by peroxynitrite [8]. Thus, the observed MAPK activation pattern by peroxynitrite may be regarded as unique in comparison to the other reactive oxygen and nitrogen species mentioned above.

The activation of p38 MAPK, JNKs and ERKs is known to lead to the induction of Fos and Jun expression which form the transcription factor AP-1 which, in turn, is capable of inducing the expression of other genes (reviewed in [4]). Thus, a new aspect of the role of peroxynitrite *in vivo* could be the modulation of gene expression.

### 3.2. Attenuation of p38 MAP kinase activation by peroxynitrite upon supplementation with selenite

When cells were grown in a medium supplemented with sodium selenite (1  $\mu$ M) for 48 h, a 14-fold increase in GPx activity was observed rising from  $4.3 \pm 0.6$  in the controls to  $54.3 \pm 1.4$  nmol/min/mg protein (means  $\pm$  S.E.M.,  $n = 3-5$ ). Supplementation of selenite (1  $\mu$ M) for 48 h had no detectable cytotoxic effects. For high concentrations of selenite (100  $\mu$ M), effects on cellular signal transduction were observed [22]; however, the concentration we used did not affect MAP kinase activation (Fig. 3A, lane 2). When peroxynitrite

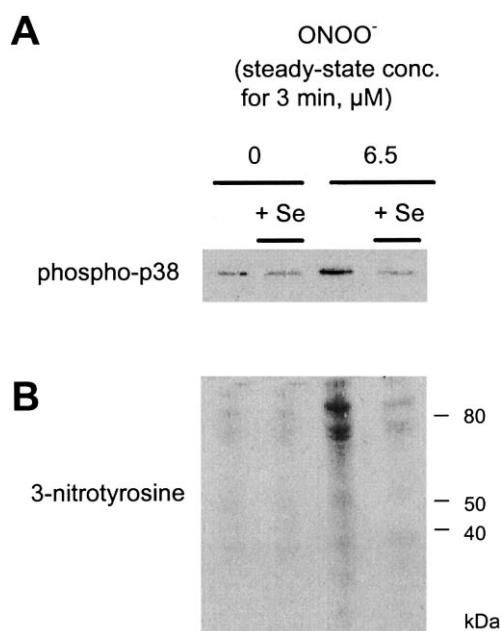


Fig. 3. Attenuation of peroxynitrite-mediated activation of p38 MAPK (A) and protein-bound tyrosine nitration (B) in rat liver epithelial cells by selenite supplementation. WB cells were grown in culture medium supplemented with selenite for 48 h. Then serum-starved cells were exposed to peroxynitrite as in Fig. 1. Cells were then immediately lysed, and phosphorylation of p38 (A) was assessed as in Fig. 1. Nitrotyrosine (B) was detected with a monoclonal anti-3-nitrotyrosine antibody (see Section 2). The results of one of three independent experiments are shown.

was infused to maintain a 6.5 μM steady-state concentration (see Section 2), cells supplemented with sodium selenite showed substantially lower activation of p38 than control cells (Fig. 3A). Nitration of protein-bound tyrosine residues, a cellular marker for peroxynitrite, was also found to be substantially lower than in unsupplemented cells (Fig. 3B).

Recently, we demonstrated that GPx catalytically protects against oxidation and nitration reactions [12]. Supplementation of sodium selenite leads to an increased synthesis of selenoproteins such as GPx [14], as verified in the present experiments (see above). Here, we show that selenite supplementation to cultured cells leads to protection against peroxynitrite-induced MAP kinase activation and against nitration of protein-bound tyrosine residues. Since selenite itself does not interact with peroxynitrite [15] this suggests a pro-

TECTIVE effect of cellular selenium-containing proteins against peroxynitrite.

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## References

- [1] Ischiropoulos, H., Zhu, L. and Beckman, J.S. (1992) Arch. Biochem. Biophys. 298, 446–451.
- [2] Kooy, N.W. and Royall, J.A. (1994) Arch. Biochem. Biophys. 310, 352–359.
- [3] Beckman, J.S. (1996) in: Nitric Oxide: Principles and Actions (Lancaster, J., Ed.), pp. 1–82, Academic Press, San Diego, CA.
- [4] Whitmarsh, A.J. and Davis, R.J. (1996) J. Mol. Med. 74, 589–607.
- [5] Lander, H.M., Jacovina, A.T., Davis, R.J. and Tauras, J.M. (1996) J. Biol. Chem. 271, 19705–19709.
- [6] Baas, A.S. and Berk, B.C. (1995) Circ. Res. 77, 29–36.
- [7] Guyton, K.Z., Gorospe, M., Kensler, T.W. and Holbrook, N.J. (1996) Cancer Res. 56, 3480–3485.
- [8] Wang, X., Martindale, J.L., Liu, Y. and Holbrook, N.J. (1998) Biochem J. 333, 291–300.
- [9] Klotz, L.-O., Briviba, K. and Sies, H. (1997) FEBS Lett. 408, 289–291.
- [10] Fisher, G.J., Talwar, S.H., Lin, J., Lin, P., McPhillips, F., Wang, Z.Q., Li, X., Wan, Y., Kang, S. and Voorhees, J.J. (1998) J. Clin. Invest. 101, 1432–1440.
- [11] Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) J. Biol. Chem. 270, 7420–7426.
- [12] Sies, H., Sharov, V.S., Klotz, L.-O. and Briviba, K. (1997) J. Biol. Chem. 272, 27812–27817.
- [13] Arteel, G.E., Mostert, V., Oubrahim, H., Briviba, K., Abel, J. and Sies, H. (1998) Biol. Chem. 379, 1201–1205.
- [14] Brigelius-Flohé, R., Lotzer, K., Maurer, S., Schultz, M. and Lest, M. (1995) Biofactors 3, 125–131.
- [15] Briviba, K., Roussyn, I., Sharov, V. and Sies, H. (1996) Biochem. J. 319, 13–15.
- [16] Koppenol, W.H., Kissner, R. and Beckman, J.S. (1996) Methods Enzymol. 269, 296–302.
- [17] Denicola, A., Freeman, B.A., Trujillo, M. and Radi, R. (1996) Arch. Biochem. Biophys. 333, 49–58.
- [18] Roveri, A., Maiorino, M. and Ursini, F. (1994) Methods Enzymol. 233, 202–212.
- [19] Klotz, L.-O., Fritsch, C., Briviba, K., Tsacmacidis, N., Schliess, F. and Sies, H. (1998) Cancer Res. 58, 4297–4300.
- [20] Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R.J. (1994) Cell 76, 1025–1037.
- [21] Dhanwada, K.R., Dickens, M., Neades, R., Davis, R. and Pelling, J.C. (1995) Oncogene 10, 1947–1953.
- [22] Stapleton, S.R., Garlock, G.L., Foellmi-Adams, L. and Kletzien, R.F. (1997) Biochim. Biophys. Acta 1355, 259–269.