

The phosphorylation state of MAP-kinases modulates the cytotoxic response of smooth muscle cells to hydrogen peroxide

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Abstract Micromolar concentrations of hydrogen peroxide induced the phosphorylation of mitogen-activated protein (MAP) kinases and a lethal response in growth-arrested smooth muscle cells (A7r5). The H₂O₂-induced phosphorylation of MAP-kinases was markedly lower in the presence of protein tyrosine kinase (PTK) inhibitors or in protein kinase C (PKC) down-regulated cells. Similarly, the toxicity of H₂O₂ was diminished by concomitant addition of either PKC or PTK inhibitors and was also lower in PKC down-regulated cells. These results are consistent with the possibility that phosphorylation of MAP-kinases is a critical event in the toxic response of cultured smooth muscle cells to H₂O₂.

Key words: Hydrogen peroxide; Cytotoxicity; Mitogen-activated protein kinase; Protein-tyrosine kinase; Protein kinase C

1. Introduction

The cytotoxic effects of hydrogen peroxide have been widely investigated in a number of different cellular systems since this oxidant is actively generated as an intermediate of a number of metabolic reactions and plays an important role in diverse pathological conditions [1]. It has been shown that H₂O₂ induces a number of cellular dysfunctions including DNA damage [2,3], depletion of glutathione [4], disruption of intracellular calcium homeostasis [5] and finally cell death. The relative contribution of each of these lesions to the cytotoxic response has not yet been identified.

In apparent contrast with the above effects, H₂O₂ induces a number of events which are also caused by mitogens. In particular, it was reported that the oxidant stimulates proto-oncogene expression [6,7] and activates PKC [8–10], PTK [11,12], protein phosphatases [12,13], MAP-kinases [14,15] as well as transcriptional activator proteins (e.g. NF-κB and AP-1) [9,16,17]. Thus, H₂O₂ is a potential mitogen, although it is important to bear in mind that it is also very toxic for the cells. Provided that sublethal concentrations of the oxidant

have the ability to stimulate the mitogenic pathways, an effect on cell proliferation may therefore be expected.

Most of the studies that have appeared in the literature, however, have utilised high concentrations of H₂O₂ and it is still unclear whether the oxidant can be considered a true mitogen. Also unclear is whether any relationship exists between the effects of the oxidant at the level of the signal transduction pathways and its toxicity.

The experiments reported in this study were aimed at investigating the effects of the interruption of the signalling pathways leading to an enhanced phosphorylation of the MAP-kinases on the lethal response of oxidatively injured growth-arrested A7r5 cells. Activation of MAP-kinases appears to be an important event in the process of H₂O₂-induced smooth muscle cell death.

2. Materials and methods

2.1. Materials

Hydrogen peroxide was purchased as a 30% solution from J.T. Baker Chemicals B.W., Deventer, The Netherlands. Dulbecco's modified Eagle medium (DMEM), minimal essential medium, foetal bovine serum (FBS), antibiotics and trypsin were from Gibco, Grand Island, NY, USA. Tissue culture materials were purchased from Falcon Labware, Becton Dickinson & Co, Basel, Switzerland. Tyrphostin A23, phorbol 12-myristate 13-acetate (PMA), and calphostin C were from LC Laboratoires, Woburn, MA, USA. Chemicals and other reagent grade biochemicals were from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Cells and cell culture conditions

Smooth muscle cells (clone A7r5, ATCC) were grown in DMEM supplemented with 25 mM sodium bicarbonate, 60 U/ml penicillin, 60 µg/ml streptomycin and 10% FBS in a humidified atmosphere containing 5% CO₂ in air, at 37°C. Media and sera used in all the experiments were from the same batch number and source. Cells between passages 2 and 12 were utilised throughout the course of this study. Cells were made quiescent by a 48 h incubation in DMEM containing 0.2% FBS.

2.3. MAP-kinase mobility shift

Quiescent cells were incubated in serum-free minimal essential medium and treated as described in the text. Cells were then rinsed with ice-cold phosphate-buffered saline (NaCl/P_i – 8 g/l NaCl, 1.15 g/l Na₂HPO₄, 0.20 g/l KH₂PO₄, 0.20 g/l KCl) containing 2 mM EDTA, and 0.5 ml lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT, 5 mM EDTA, 10 mM EGTA, 10% (v/v) glycerol, 0.25 M sucrose, 1% (w/v) Triton X-100, and freshly prepared 1 mg/l PMSF and E64, 500 µg/l chymostatin, leupeptin, antipain and pepstatin, 50 mM sodium fluoride and 100 µM sodium orthovanadate) was added. Cell lysates were prepared by scraping, sonication and passes through a 25-gauge needle. After centrifugation for 30 min at 100 000 × g, aliquots were taken for protein determination using the Pierce bicinchonic acid assay. Total cell lysate (25–35 µg protein) was electro-

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Abbreviations: MAP-kinase, mitogen-activated protein kinase; PTK, protein-tyrosine kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle medium; FBS, foetal bovine serum; NaCl/P_i, phosphate-buffered saline

phoresed on a 10% polyacrylamide gel, blotted onto poly(vinylidene difluoride) transfer membranes (DuPont, NEN Research Products) and incubated with 0.1 µg/ml anti-MAP kinase R2 rabbit polyclonal antibody (Erk1-CT, Upstate Biotechnology Incorporated, NY, USA). Immune complexes were detected with the enhanced chemiluminescent (ECL) Western blotting detection system (Amersham International plc, Bucks, UK).

2.4. Cytotoxicity assays

Quiescent cells were incubated in serum-free minimum essential medium and treated as detailed in the text. Cytotoxicity was determined by measuring cytoplasmic lactate dehydrogenase (LDH) release. After exposure for 5 h to hydrogen peroxide in the absence or presence of various modulators of cytotoxicity (see Section 3), the culture medium was removed and the cell monolayers were rinsed with NaCl/P_i. Subsequently, the cells were lysed for 30 min at room temperature with NaCl/P_i containing 0.5% Triton X-100. LDH activity was measured in the culture medium as well as in the cell lysates as previously described [18]. LDH release was expressed as the percentage of the total enzyme activity (i.e. that released from the cells into the culture medium plus that associated with the cells).

3. Results and discussion

The effect of H₂O₂ on MAP-kinase phosphorylation was investigated in growth-arrested A7r5 cells. As illustrated in Fig. 1, exposure to 150 µM H₂O₂ for 1.5 h resulted in increased phosphorylation of both MAPK1 and MAPK2, as indicated by a shift to higher molecular mass of the protein bands. This response was significantly higher than that observed after treatment with 100 nM PMA (not shown). PKC down-regulation, achieved by incubating the cells for 24 h with 1 µM PMA, diminished – but did not abolish – the extent of the H₂O₂-induced MAP-kinase phosphorylation. The fact that the PTK inhibitor tyrphostin A23 (20 µM) also reduced this response indicates that this effect of H₂O₂ is dependent on the activation of both PKC and PTK. The interpretation of these results is consistent with previous work performed in this [9,10] as well as in other [8,11,12] laboratories demonstrating that H₂O₂ efficiently stimulates PKC and PTK.

To investigate whether tyrosine phosphorylation is involved in H₂O₂-induced smooth muscle cell killing, quiescent A7r5 cells were treated for 5 h with increasing concentrations of H₂O₂ in the absence or presence of the PTK inhibitor tyrphostin A23 and then analysed for cytotoxicity using the LDH release assay. As illustrated in Fig. 2A, treatment with the oxidant resulted in a concentration-dependent induction of cell death and this response was linear over H₂O₂ concentrations ranging from 37.5 to 100 µM. Addition of either 20 µM tyrphostin A23 2 min prior to exposure to the oxidant reduced cell killing, thus suggesting a potential role for PTK in this response.

We next studied the effect of depletion of the conventional and novel PKC isoforms in the toxic response of A7r5 cells to H₂O₂. PKC-α, -δ and -ε isoforms were depleted by down-regulation while the ζ-isoform was not affected [9,10]. As illustrated in Fig. 2B, cell killing promoted by H₂O₂ was significantly reduced by prolonged exposure to PMA. The results of Fig. 2A show that the PKC inhibitor calphostin C, at a concentration (130 nM) previously shown [10] to abolish PKC activity stimulated by H₂O₂, efficiently reduced the toxicity elicited by the oxidant. Importantly, under the experimental conditions utilised in this study, neither tyrphostin A23 nor calphostin C provoked LDH leakage into the culture medium

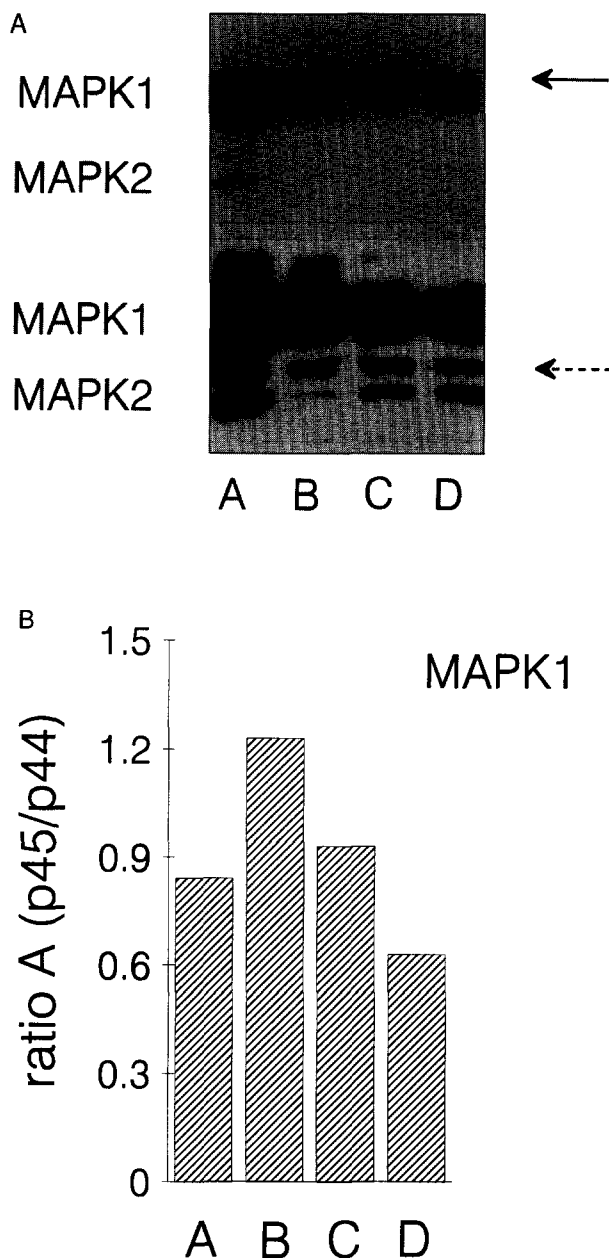


Fig. 1. The effect of hydrogen peroxide on MAP-kinase phosphorylation in growth-arrested A7r5 cells. (A) Quiescent cells (control, A) were treated for 1.5 h in the absence (B) or presence (C) of 20 µM tyrphostin A23. The effect of 150 µM H₂O₂ in PKC-downregulated cells is also shown (D). PKC downregulation was achieved by 24 h treatment of cells with 1 µM PMA. Following the incubations, total cell lysates were prepared and MAP kinase activity was examined by mobility shift assay as described in Section 2. The 44-kDa form of MAP-kinase is designated MAPK1 while the 42-kDa form is designated MAPK2. The solid and broken arrows indicate the activated (phosphorylated) forms of MAPK1 and MAPK2, respectively. The upper and lower gels differ in the length of exposure after the ECL reaction. (B) Above autoradiographies were scanned by densitometry and the relative signal ratio between activated (p45 and p43) and non-activated (p44 and p42) forms of MAPK1 and MAPK2 expressed as arbitrary units of absorbance (A) are shown. A value equal to unity corresponds to identical intensity of the activated and non-activated bands.

(not shown). Furthermore, the cytoprotection afforded by the PTK or PKC inhibitors was not the consequence of a scavenging effect since, under similar experimental conditions,

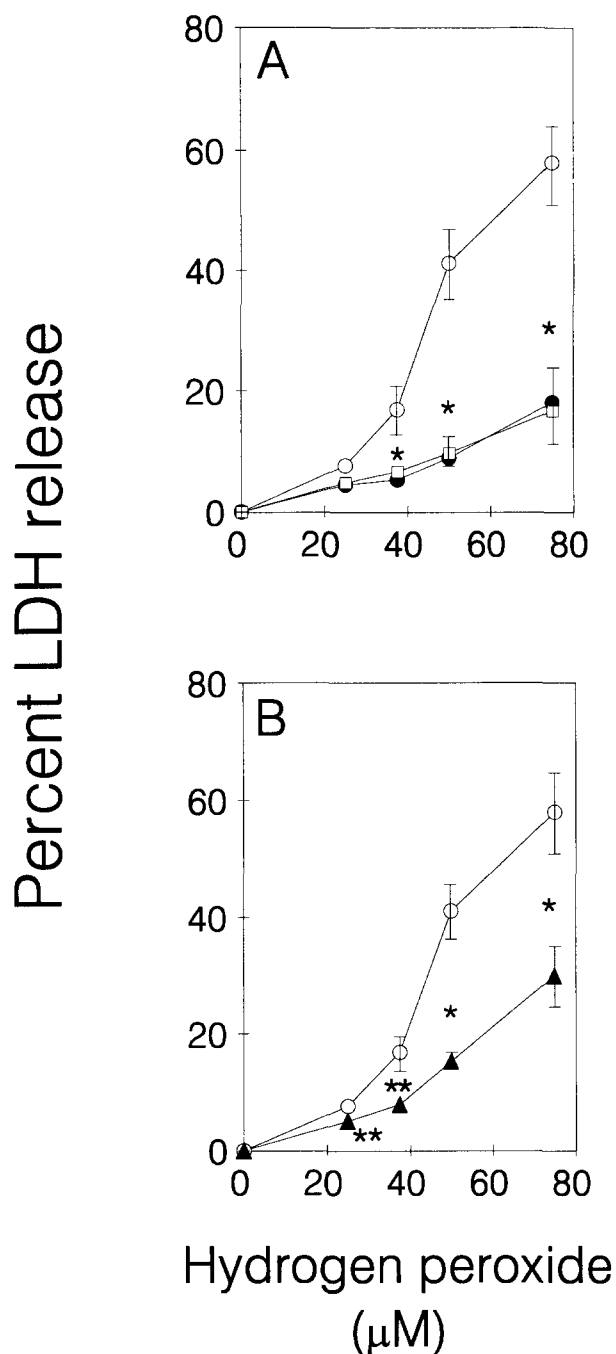


Fig. 2. The effect of PKC and PTK inhibitors and PKC-down-regulation on the toxicity of hydrogen peroxide in growth-arrested A7r5 cells. (A) Growth-arrested A7r5 cells were treated for 5 h with increasing concentrations of hydrogen peroxide in serum-free minimal essential medium in the absence (○) or presence of 20 μM tyrphostin A23 (●) or 130 nM calphostin C (□). (B) Cells were exposed for 24 h (during serum starvation) to 1 μM PMA and then treated for 5 h with increasing concentrations of H₂O₂ in serum-free minimal essential medium. (○) Control; (▲) downregulated cells. After treatment, the level of cytotoxicity was estimated using the LDH release assay that was performed as detailed in Section 2. Values represent the means ± S.E.M. calculated from 3–7 experiments, each performed in duplicate. **p* < 0.005, ***p* < 0.01 vs treatment with hydrogen peroxide alone.

they did not reduce the extent of DNA single strand breakage generated by H₂O₂ (not shown). The fact that inhibition of either PTK or PKC was associated with a marked reduction

in cell killing, along with the observation that depletion of the conventional and novel PKC isoforms also reduced the lethal response to the oxidant, would suggest that activation of PKC and PTK are critical events in the development of the toxic response of growth-arrested A7r5 cells to challenge with H₂O₂. It would therefore appear that the processes of H₂O₂-induced stimulation of MAP-kinase activity and cytotoxicity are similarly controlled by PKC and PTK.

The results presented in this paper extend our previous work in which we demonstrated that H₂O₂ stimulates DNA synthesis in growth-arrested A7r5 cells and that this process was not followed by cell proliferation but, rather, by cell death [10]. We now report that inhibition of PKC or PTK, while reducing the effects of H₂O₂ on MAP-kinase phosphorylation, also reduces the toxicity promoted by the oxidant. Thus, the MAP-kinase pathway, which is normally activated by diverse mitogenic stimuli [19–21], may also be involved in the expression of the lethal response in cells intoxicated with H₂O₂.

In conclusion, our results suggest that the lethal effects promoted by H₂O₂ in growth-arrested smooth muscle cells might not simply be the consequence of the deleterious effects which have been thus far described in a number of different cell lines and postulated to be the cause of the toxicity of H₂O₂. Rather, it would appear that cell death is mediated or at least modulated by specific signal transduction pathways. It is conceivable that in cells exposed to an agent which generates an array of toxic lesions, and concomitantly initiates the biochemical events which finally lead to DNA synthesis, an active signalling pathway may promote the onset of cell death with the specific aim of preventing cell proliferation. The MAP-kinases could well represent a nodal component of this/these signalling pathway(s).

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