ELSEVIER

Biochemical Pharmacology 63 (2002) 163–170

Biochemical Pharmacology

Role of redox status on the activation of mitogen-activated protein kinase cascades by NSAIDs

Ana Maria Lennon, Martine Ramauge, Michel Pierre*

U 488 INSERM, 80 rue du Général Leclerc, 94276 Le Kremlin-Bicêtre, France

Received 8 June 2001; accepted 25 September 2001

Abstract

High concentrations of non steroidal antiinflammatory drugs (NSAIDs) exert preventive effects against carcinogenesis. Their molecular mechanism of action remains to be elucidated. Based on previous reports with salicylate, we have made the hypothesis that various NSAIDs can activate the mitogen-activated protein kinases (MAPK). Moreover, we tested the idea that NSAIDs act by increasing the effects of oxidative stress. We report that in human colorectal carcinoma cells NSAIDs stimulated the three families of MAPK, extracellular regulated kinases, c-Jun N-terminal kinases, p38 MAPK and that this stimulation is prevented by *N*-acetyl cysteine. In cultured astrocytes, a biological system less sensitive to oxidative stress, we show that a short treatment by NSAIDs strongly activated the three MAP kinases in the presence of H_2O_2. A 25 μM H_2O_2, unable to stimulate by itself the MAP kinases, promote an almost complete activation of MAP kinases in the presence of NSAIDs. The activation of MAP kinases by H_2O_2 and NSAIDs was suppressed by quinone reductase inhibitors, suggesting that ''redox cycling'' was involved in the activation mechanisms of MAP kinases by H_2O_2 and NSAIDs. The mobility on SDS–PAGE of the apoptosis signal-regulating kinase, which activates C-Jun N-terminal kinases and p38 MAPK cascades, was reduced by H_2O_2 and NSAIDs, suggesting, that H_2O_2 and NSAIDs activated apoptosis signal-regulating kinase by increasing its state of phosphorylation. In conclusion, we demonstrate that various NSAIDs can activate the three families of MAP kinases and that this activation depends on the presence of reactive oxygenated species. These results give a new insight into the mechanism of the action of NSAIDs.

Keywords: Astrocytes; NSAIDs; Oxidative stress; MAP kinases; ASK1; Ros

1. Introduction

Three mitogen-activated protein kinases (MAPK) families have been identified in mammalian cells: the extracellular regulated kinases (Erks), the c-Jun N-terminal kinases (Jnks) and the p38 MAPKs (for review see Murga *et al.* 1999 [1]). MAPKs transduce information into the different compartments of the cell. The three MAPK families have different downstream targets that they phosphorylate on serine and threonine residues located adjacent to proline residues. The three MAPK families are activated by MAPK kinase (MAPKK) upstream dual-specificity kinases, phosphorylating threonine and tyrosine residues in a threonine-X-tyrosine site specific for each MAPK family. MAPKKs are activated by specific MAPKKKs (Rafs, MEKK1, apoptosis signal-regulating kinase (ASK1), MLKs, TAK, etc.). The three MAPK families are activated in response to various stimuli. Erk family is activated preferentially by growth factors usually acting through a Ras-Raf-dependent cascade, while Jnk and p38 MAPK families are preferentially activated by stress factors and cytokines of the transforming growth factor-α (TNFα) family. However, the specificity of activating stimuli is relative, depending on the cell type. For example, in astrocytes Erk is strongly activated by a number of stress factors including oxidative stress ([2] and unpublished observations). Sensing of the stress and its mechanisms of coupling to MAPK cascades are still not understood.

* Corresponding author. Tel.: +33-1-49591834; fax: +33-1-45211940.
E-mail address: pierre@kb.inserm.fr (M. Pierre).

Abbreviations: NSAIDs, non steroidal antiinflammatory drugs; HT29, human colorectal carcinoma cells; MAPK, mitogen-activated protein kinases; Erks, extracellular regulated kinases; P-Erk, active Erk; Jnks, c-Jun N-terminal kinases; P-Jnk, active Jnk; P-p38, active p38 MAPK; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; ASK1, apoptosis signal-regulating kinase; NAC, *N*-acetyl cysteine; Ros, reactive oxygenated species; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MBP, myelin basic protein; PAGE, polyacrylamide gel electophoresis; TNFα, transforming growth factor-α; ARE, antioxidant responsive element.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00826-7

Salicylate, one NSAID has been reported to activate p38 MAPK in normal human fibroblasts [3] and Jnk and p38 MAPK in human colorectal carcinoma cells (HT29) and COS cells [4]. Salicylate, like other non steroidal antiinflammatory drugs (NSAIDs) such as aspirin and sulindac, are effective in reducing colon tumors in genetically susceptible humans [5] and rodents [6–8]. Epidemiological studies have demonstrated that NSAIDs used in the general population is associated with a reduced risk of colon cancer death [9]. NSAIDs also inhibit the growth of other types of cancer cells [10–12]. These anti-tumor effects and the effects on Jnk and p38 MAPK require drug concentrations higher than those inhibiting the cycloxygenases cox-1 and cox-2. We do not know whether the activation of these MAPKs plays a role in the anti-tumor effects of these drugs. On the other hand, high concentrations of NSAIDs such as salicylate, aspirin and sulindac inhibit NFκB activation [13–16], and this inhibition can contribute to their anti-tumor effect. Some preliminary observations also indicate that NSAIDs could be used for treatment of Alzeihmer and other neurodegenerative diseases [17–20]. All these observations raise the question of the role of MAP kinases in these diseases: stimulation of cell death or protection against cellular damages?

The astrocytes constitute nearly half of the cells of the brain and play a fundamental role to protect the brain. It is important to understand how these cells respond to biological extracellular signals and drugs. We studied for several years in these cells, the mechanisms of MAP kinases activation by growth factors [21], hormones [22] and stress factors [2], We have now examined whether salicylate and others NSAIDs can activate MAP kinases in these cells. We found that the three families of MAP kinases and ASK1 are activated by all the NSAIDs tested, but this activation was dependent on the redox state of the cells. Indeed, an ''oxidized state'' of the cell potentiates strongly the activation of MAP kinases by NSAIDs. The redox state of the cell is believed to be controlled by molecule(s) involved in ''redox cycling'' [23]. In ''redox cycling'', reactive quinones catalyse the oxidation of intracellular sulphydryls. According to this view, we find that NSAIDs activate the three families of MAP kinases by a mechanism sensitive to the quinone reductase inhibitor, menadione.

2. Material and methods

2.1. Materials

Sprague–Dawley rats were from our own breeding. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and Ham F-12 culture mediums were from Life Technology. Myelin basic protein (MBP), menadione, *N*-acetyl cysteine (NAC), sulindac, salicylate and diclofenac were from Sigma. Antibodies against active forms of Erk, Jnk and p38 MAPK were purchased from Promega. Antibody against ASK1 was from Santa Cruz Biotechnology. Antibody against rat thioredoxin was from IMCO. Horseradish peroxidase-conjugated secondary antibody was from Vector Laboratories. PVDF membranes were furnished by NEN. Chemiluminescence systems were purchased from Amersham and NEN. γ^{32}P-ATP was from Amersham. In Situ Cell Detection Kit, was from Roche Molecular Biochemicals. All others products were of quality grade.

2.2. Methods

2.2.1. Cell cultures

Cells were prepared as previously described [24]. Briefly, astroglial cells were obtained from the cerebral hemispheres of 2-day-old Sprague–Dawley rats, plated in 10 cm^2 dishes and grown at 37°, 5% CO_2, in DMEM, supplemented with: 6 g/L glucose, 2.4 g/L sodium bicarbonate, antibiotics (100 U/mL penicillin, 100 μg/L streptomycin and 0.25 μg/L amphotericin B) and 10% FCS. The medium was changed every 2–3 days until the cells reached confluence at about 10 days. The cells were further cultured for a week, with daily changes, in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 5.2 g/L glucose, 1.8 g/L sodium bicarbonate and the antibiotics listed above. Ninety-five percent of these cells contained, as we have previously described [25], the immunoreactive glial fibrillary acidic protein, a specific marker of astrocytes.

HT29 cells were cultured at 37°, 5% CO_2, in DMEM containing 4.5 g/L glucose, 2 mM glutamine and 10% FCS. The medium was changed every 2–3 days. Before the experiments, cells were serum-starved for 24 hr in DMEM with 0.25% FCS.

2.2.2. MAP kinase assay

The cultures were treated with different agents and at the end of the incubation period, the culture medium was removed and the cells were rinsed once with 1 mL of the iced kinase buffer: (80 mM 2-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $MgCl_2$). The cells were scraped off into 300 μL iced kinase buffer containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 50 μg/mL aprotinin, 4 μg/mL leupeptin, 10 μg/mL antipain, 1 μg/mL trypsin inhibitor, 1 mM benzamidine and 10 μg/mL pepstatin) and 1 mM of the phosphatase inhibitor orthovanadate (Na_3VO_4). The cell suspension was then sonicated for 5 s on ice and centrifuged at 105,000 *g* for 35 min at 4°. The supernatants were aliquoted and stored at −80° until use.

MAPK (Erk) activity was assayed as previously described [2]. Briefly, aliquots of the cell supernatants were incubated for 10 min at 30° in 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 20 μM γ^{32}P-ATP (2 μCi), 17 μg of MBP and 10 μg/mL of the peptide inhibitor of cAMP-dependent

protein kinase. The reaction was initiated by adding an aliquot of the supernatants and stopped by spotting 40 μL of the reaction mixture onto Whatman 3MM paper. ^{32}P incorporation into proteins was determined after paper washing.

2.3. Immunoblotting

The cultures were treated with different agents and at the end of the incubation period, cells were scraped off in Laemmli buffer [26]. Identical amounts of proteins from each sample were separated by SDS–polyacrylamide gel electophoresis (PAGE), then transferred onto PVDF membranes by semidry transfer. The membranes were blocked with 3% bovine serum albumin in PBS–Tween 0.1%.

Immunodetections were performed by incubating the PVDF membrane with the following antibodies: anti-active Erk (P-Erk) (1/5000), anti-active Jnk (P-Jnk) (1/5000), anti-active p38 MAPK (P-p38) (1/2000) and anti-ASK1 (1/1000) in PBS–Tween 0.1% and 1% bovine serum albumin. Detection of antigen–antibodies complexes was performed with horseradish peroxydase-conjugated secondary antibody and the revelation was made by chemiluminescence reaction. All the experiments were repeated at least three times.

2.3.1. Cell apoptosis assay

The cells were treated with the different agents for 24 hr and then fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for detecting DNA fragmentation was performed using the In Situ Cell Detection Kit, Fluorescein. The cell nuclei were stained with DAPI and total and apoptotic cells were counted using a fluorescent microscope. The results were expressed as percentage of apoptotic cells.

2.3.2. Protein determination

The protein content of the samples in Laemmli buffer was determined by the method of Mc Knight [27], using bovine serum albumin as standard. The protein content of cell sonicates was determined by the method of Bradford [28].

3. Results

3.1. Ros requirement for activation of MAP kinases by NSAIDS

In our hands, salicylate alone activated Jnk and p38 MAPK in HT29 cells as previously described by Schwenger *et al.* [3,4]. However, in contrast to their results we observed that salicylate also promoted the activation of Erk. Indeed, the activation of the three families of MAP kinases was detectable after 30 min and was maximal at 120 min (Fig. 1). Activation of p44 Erk, one isoform of p38 MAPK (corresponding to the faster migrating isoform in the astrocytes) and one isoform of P-Jnk (corresponding to the slower migrating isoform in the astrocytes) were detected. We believe that in HT29 cells these activations involve the generation of reactive oxygenated species (Ros) due to the strong metabolism in these cells, because NAC completely prevented these activations (Fig. 1). Sulindac and diclofenac promoted a weaker activation of the MAP kinases in HT29 cells (not shown).

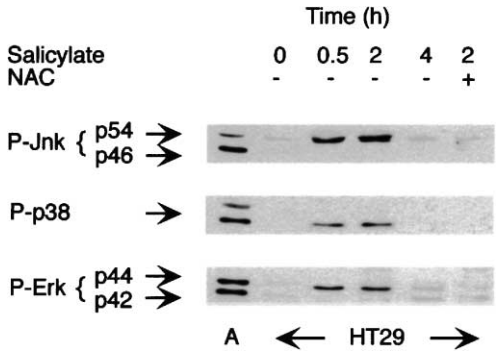

Fig. 1. Effect of salicylate and *N*-acetyl cysteine on the activation of MAP kinases in HT29 cells. HT29 cells maintained overnight in low-serum-medium were treated for various times with salicylate (50 mM) and when indicated with NAC (20 mM). Dishes were scraped off in kinase buffer, sonicated and centrifuged for 25 min at 105,000 *g*. Identical amounts of cytosolic proteins were analyzed by Western blot to detect the activation of Jnk, p38 MAPK and Erk as described in Section 2. Figure shows the immunoblots obtained after ECL. (**A**) Sample of astrocytes treated with H_2O_2 (200 μM, 20 min).

Addition of 200 μM sulindac or 50 mM salicylate or 75 μM diclofenac (concentrations able to inhibit cell proliferation [5–7] and [9–11]), to primary cultures of astrocytes from 0 to 90 min, did not activate, or only weakly, Erk, Jnk and p38 MAPK as shown in Figs. 2–4. Since MAP kinases activation by NSAIDs in HT29 cells appears to depend on the presence of Ros, we added Ros (200 μM H_2O_2) to cultured astrocytes, in the presence of sulindac, salicylate and diclofenac. The NSAIDs strongly increased the rate of activation of MAP kinases by H_2O_2. This effect was easily visible for Erk (Fig. 2) by measuring MBP phosphorylation or by monitoring Erk phosphorylation with the antibody directed towards the active form of Erk (Fig. 4). NSAIDs also increased the phosphorylation of the Jnk and p38 MAPK induced by H_2O_2 (Figs. 3 and 4). Activated forms of p44 and p42 Erk, two isoforms of Jnk and two isoforms of p38 MAPK were seen. Maximum activation promoted by NSAIDs in presence of H_2O_2 was generally obtained after 15 min (Figs. 2 and 3). This effect was almost completely suppressed by 20 mM NAC (Fig. 4) indicating that the effect of NSAIDs depended on the redox state of the cells. As 200 μM H_2O_2 elicited a strong activation of MAP kinases, which is almost complete after 45 min of cell treatment in most of the cultures but which is more rapid in some cases ([2] and Fig. 5), we speculated

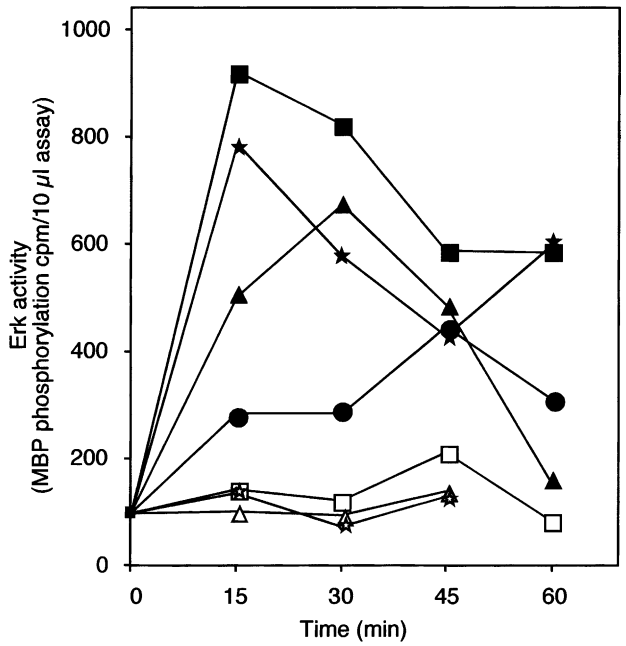

Fig. 2. Kinetics of the effect of sulindac, salicylate and diclofenac on H_2O_2 induced-Erk. Sulindac (200 μM), salicylate (50 mM) or diclofenac (75 μM) were added to cultured astrocytes 5 min before H_2O_2 (200 μM). Incubations were ended at various times after H_2O_2 addition and Erk activity was assayed as described in Section 2. The values are the mean of two determinations and the curve is representative of three different experiments. ((□) Sulindac, (☆) salicylate, (△) diclofenac, (■) sulindac + H_2O_2, (★) salicylate + H_2O_2, (▲) diclofenac + H_2O_2 and (●) H_2O_2 alone).

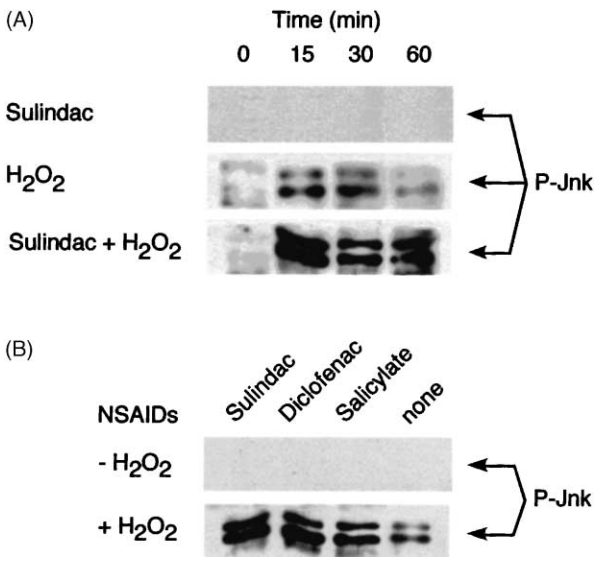

Fig. 3. Effect of sulindac, salicylate and diclofenac on H_2O_2 induced Jnk activation. (**A**) Astrocytes were treated with sulindac (200 μM), and H_2O_2 (200 μM), for the indicated times. (**B**) Sulindac (200 μM), salicylate (50 mM), or diclofenac (75 μM) were added to cultured astrocytes 5 min before H_2O_2 (200 μM) for 15 min. Dishes were then scraped off in Laemmli buffer and Jnk activation was evaluated by Western blot as described in Section 2. Figure shows the immunoblot obtained after ECL.

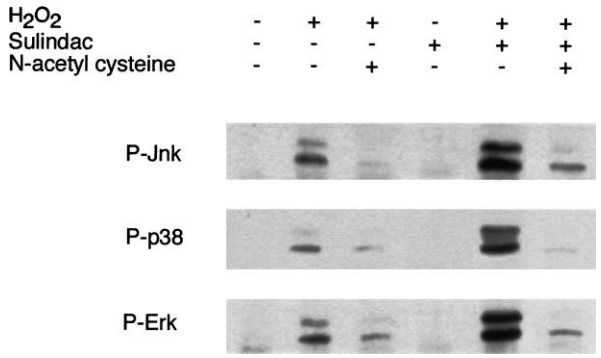

Fig. 4. Suppression by *N*-acetyl cysteine of the H_2O_2 induced-MAP kinases potentiated by sulindac. Sulindac (200 μM) was added or not 5 min before H_2O_2 (200 μM, 15 min). When indicated, NAC (20 mM) was added 30 min before the other compounds. Dishes were scraped off in Laemmli buffer and the activation of Jnk, p38 MAPK and Erk was evaluated by Western blot as described in Section 2. Figure shows the immunoblots obtained after ECL.

that the ability of NSAIDs to amplify MAP kinases activation would be stronger at lower concentrations of H_2O_2.

3.1.1. Dependence of MAP kinases activation by NSAIDs on H_2O_2 concentration

To test our hypothesis, astrocytes were exposed to increasing concentrations (25–200 μM) of H_2O_2 for 15 min in absence or presence of sulindac. Activation of Erk, Jnk and p38 MAPK elicited by this treatment is shown in Fig. 5. As reported above, sulindac alone did not activate the three classes of MAP kinases. In this experiment, H_2O_2 alone (200 μM), promoted an almost complete activation after 15 min whereas H_2O_2 below 100 μM had only a small effect on the activation of MAP kinases. In contrast, in the presence of sulindac, activation of MAP kinases (Erk, Jnk, p38 MAPK) was almost complete at 25 μM H_2O_2. In astrocytes treated with 200 μM H_2O_2 alone, the rate of MAP kinases activation can be slower than in this experiment (for Erk see Fig. 2 and for Jnk Fig. 3A, not shown for p38 MAPK). Even, in this case maximal activation by H_2O_2 + NSAIDs was already obtained at 15 min (for Erk see Fig. 2 and for Jnk Fig. 3A).

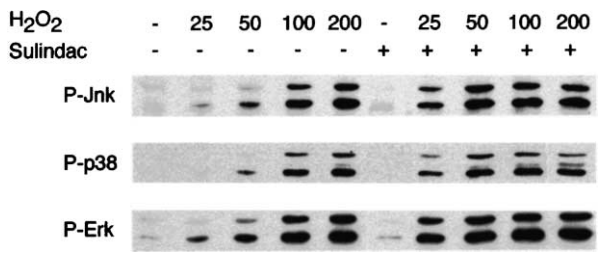

Fig. 5. Dependence of sulindac effect on H_2O_2 concentration. Astrocytes were treated or not with sulindac (200 μM), 5 min before adding increasing concentrations (0–200 μM) of H_2O_2. After 15 min, dishes were scraped off in Laemmli buffer and the activation of Jnk, p38 MAPK and Erk was evaluated by Western blot as described in Section 2. Figure shows the immunoblots obtained after ECL.

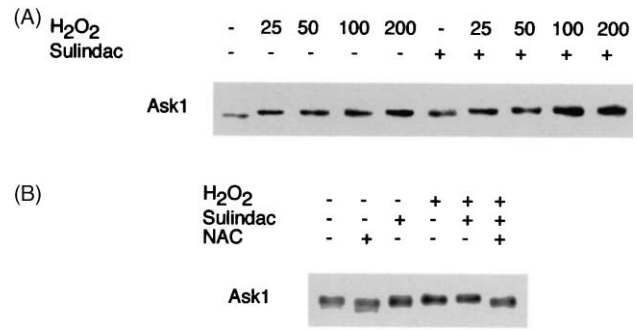

Fig. 6. Effect of H_2O_2, sulindac and NAC on ASK1 mobility on SDS–PAGE. (A) Astrocytes were treated or not with sulindac (200 μM), 5 min before the addition of increasing concentrations (0-200 μM) of H_2O_2 for 15 min. (B) Astrocytes were treated or not with sulindac (200 μM) 5 min before the addition of H_2O_2 (200 μM) for 20 min. When indicated, NAC (20 mM) was added, 30 min before the other compounds. Dishes were scraped off in Laemmli buffer and ASK1 was detected by Western blot as described in Section 2. Figure shows the immunoblots obtained after ECL.

3.1.2. Activation of ASK1 by NSAIDs

Since the MAPKKK, ASK1, has been shown to be activated by H_2O_2 [29], we looked for activation of ASK1 by NSAIDs. We found that treatment of astrocytes by increasing concentrations of H_2O_2 (0–200 μM) reduced the rate of migration of ASK1 on SDS–PAGE (Fig. 6) as observed for a number of protein kinases including MAP kinases. Sulindac alone also reduced the rate of migration of ASK1. As reported above for MAP kinases, NSAIDs increased the sensitivity to H_2O_2. In the presence of both, sulindac and H_2O_2 the mobility of ASK1 decreased more. NAC prevented the effect of sulindac + H_2O_2 and alone also increased the rate of migration of ASK1 in extracts of unstimulated cells (Fig. 6B). These observations suggest that oxidative stress, as for other protein kinases, activates ASK1 by modulating its phosphorylation state and that NSAIDs can increase the rate of ASK1 phosphorylation.

3.1.3. Suppression by the quinone reductase inhibitor, menadione, of the activation of MAP kinases by oxidative stress

Cross *et al.* [23], have reported that quinone reductase inhibitors can prevent Jnk activation by sorbitol, anisomycin, TNFα and UVC, but not by heat shock and TPA. The quinone reductase inhibitors prevent Jnk activation likely by blocking the oxidation of intracellular sulphydryls by reactive quinones [30]. This oxidation of sulphydryls by reactive quinones is termed "redox cycling" [31] and might be one element linking stress factors and MAP kinases. The quinone reductase inhibitor, menadione also inhibits the reduction of sulindac [32].

Quinone reductase inhibitors, menadione and dicoumarol reduced the effect of 200 μM H_2O_2 on the activation of MAP kinases (not shown). In our hands, with cultured astrocytes, menadione was the strongest inhibitor of the activation of MAP kinases by oxidative stress. Consequently, menadione was used for subsequent experiments.

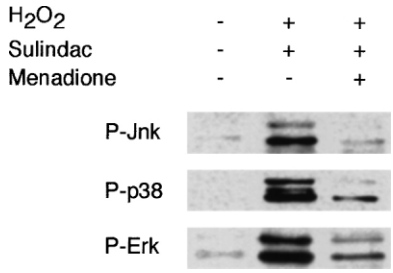

Fig. 7. Inhibition by menadione of the H_2O_2 induced-MAP kinases activation. Astrocytes were treated with sulindac (200 μM), in the presence or absence of menadione (100 μM) 5 min before the addition of H_2O_2 (50 μM) for 20 min. Dishes were scraped off in Laemmli buffer. The activation of Jnk, p38 MAPK and Erk was evaluated by Western blot as described in Section 2. Figure shows the immunoblots obtained after ECL.

We examined whether menadione prevents activation of MAP kinases by 50 μM H_2O_2 and sulindac. Fig. 7 shows that menadione effectively blocks the activation of Erk, Jnk and p38 MAPK by H_2O_2 and sulindac. Similar results were obtained with salicylate or diclofenac (not shown). These observations suggest that "redox cycling" plays a key role in the activation of MAP kinase cascades by NSAIDs and oxidative stress.

3.1.4. Effects of NSAIDs and H_2O_2 on cell death

As we have observed previously for 200 μM H_2O_2 [2], sulindac alone or associated with 200 μM H_2O_2 does not promote significant cell death in astrocytes. DNA content by dish was not modified after 24 hr (not shown). Sulindac alone, 200 μM H_2O_2 and their association did not increase after 24 hr, the number of apoptotic cells. In a typical experiments the percentage of apoptotic cells were: control dishes: $9.55 \pm 1.8\%$; sulindac (200 μM): $7.69 \pm 1.53\%$; H_2O_2 (200 μM): $9.09 \pm 0.53\%$; H_2O_2 (200 μM) +sulindac (200 μM): $11.84 \pm 1.16\%$; $n = 5$.

4. Discussion

From the present work, we propose that NSAIDs can activate the three families of MAP kinases in different cells provided Ros be present. Indeed, using astrocyte cultures, we have shown that NSAIDs (sulindac, salicylate and diclofenac) can strongly increase the rate of activation of the three families of MAP kinases: Erk, Jnk and p38 MAPK. Maximal activation can be obtained within 15 min. However, this effect of NSAIDs depends on the redox state of the cells, since a low concentration of H_2O_2 (25 μM) must be added to the cells to obtain the activation of MAP kinases. This low H_2O_2 concentration does not activate by itself the MAP kinase cascades. We also have shown that the MAPKKK, ASK1, which can mediate the activation of Jnk and p38 MAPK [33] and is activated by H_2O_2, displays a shift on SDS–PAGE, in extracts of astrocytes treated by NSAIDs and H_2O_2. This shift is

prevented when NAC is present in cell culture medium. We conclude that this shift is associated with ASK1 activation and that it is probably due to changes in the phosphorylation state of ASK1.

We propose that NSAIDs increase the oxidation of sulphydryls groups of proteins such as thioredoxin, which are believed to be involved in the sensing mechanism of the redox state. Reduced thioredoxin can bind ASK1 to form an inactive complex [29]. Oxidation of thioredoxin by ''redox cycling'' might promote thioredoxin release from the complex and ASK1 activation to stimulate Jnk and p38 MAPK cascades. Consequently, thioredoxin and ASK1 might constitute a link between a system sensing the redox state of the cell and signaling pathways. In our biological system, this hypothesis is supported by the inhibition by NAC and by menadione and dicoumarol of the H_2O_2-induced activation of MAP kinases. NAC is a scavenger of free radicals that improves the accumulation of reduced peptides containing thiols like glutathion [34]. Menadione and dicoumarol share the property to inhibit the quinone reductase, NQO1 [23,35], an enzyme regulating reactive quinones implicated in the process termed ''redox cycling'' [30], in which reactive quinones catalyze the oxidation of intracellular sulphydryls. Menadione, can also generate oxygen radicals when reduced by cellular reductases that generate highly reactive semiquinone intermediates [36]. But, it is likely that the generation of oxygen radicals from menadione is completely masked in astrocytes by the inhibition of the ''redox cycling''. Otherwise, immunoprecipitation of rat thioredoxin (not shown), in contrast with the observations of Saitoh *et al.* [29], does not confirm an interaction, regulated by oxidative stress, of thioredoxin with ASK1. In this way, Liu *et al.* [37] reported an effect of TNFα on the complex ASK1-thioredoxin, but failed to observe an effect of H_2O_2 on this complex. The role of thioredoxin is still not well understood and it is likely that thioredoxin oxidation is not the single reaction promoted by oxidative stress which is implicated in the activation of MAP kinases.

ASK1 activation can explain Jnk and p38 activation, but not the activation of Erk that we also observe in astrocytes treated by NSAIDs and H_2O_2. Some observations suggest that activation of the Erk cascade by Ros involves the activation of tyrosine kinases. The protein-tyrosine phosphatase 1B that is regulated by thioredoxin, may play a role in these process [38]. These activation mechanisms involved during the first 20 min could be prolonged by other molecular mechanisms involving PLA2 and lipoxygenases, as described in a previous work [2].

ASK1 means apoptosis signal-regulating kinase, but its role is not limited to initiate apoptosis. ASK1-dependent activation of Jnk and p38 MAPK regulates the expression of genes involved in the defense and the survival of cells like hsp27 [39], antioxidant and phase II detoxifying enzymes [40]. These antioxidant and phase II detoxifying enzymes include NAD(P)H:quinone oxidoreductase, glutathion S-transferase. The induction of these enzymes occurs at the transcriptional level through an antioxidant responsive element (ARE) and may be mediated by Erk cascade [41], while p38 MAPK cascade inhibits the ARE-mediated gene expression [40]. NSAIDs have been reported to induce Quinone reductase and glutathion S-transferase [42] and we suggest that they may promote these inductions by activating MAP kinases pathways as described in this paper. Activation of MAP kinase cascades also plays a role in NFκB activation [43] but, in astrocytes, NSAIDs although they can activate MAP kinases, inhibit NFκB activation [16]. In our cultured astrocytes, activation of MAP kinases by H_2O_2 + NSAIDs does not initiate apoptosis suggesting that the activation of MAP kinases is rather implicated in survival mechanisms.

In HT29 cells, that display a strong metabolism and have probably a regulation of the redox state different from astrocytes, we have observed that salicylate alone induces a slow activation of the three MAP kinases families as reported for Jnk and p38 MAPK by Schwenger *et al.* [4]. In these cells, it is possible that salicylate promotes a progressive accumulation of reactive oxygen derivatives contributing to MAP kinases activation. This hypothesis is supported by the inhibitory effect of NAC on the salicylate-induced MAP kinases activation. The possible induction of reactive oxygen derivatives by salicylate may involve the known action of NSAIDs on mitochondria [44–46]. Differences in the redox systems of each cell type may explain the differences in MAP kinases activation by NSAIDs observed between rat astrocytes and HT29 cells. Moreover, other MAPKKKs (MEKK1, TAK1, etc.) than ASK1 can be involved in the activation of Jnk and p38 MAPK. In this respect, it has just been reported that the activation by exisulind of the protein kinase G leading to JNK1 activation, occurs by direct phosphorylation of MEKK1 [47]. The content of MAPKKKs and MAPKKs differs probably between the cell types and consequently, cell responses to stress may be different.

It is very important to understand the regulation of the activation of MAP kinase cascades by stress, since they can contribute either to cell survival or cell death. In cancer treatments, it may be advantageous to improve tumor cell death, but other cells must be preserved to avoid negative side-effects of the treatment. In neurodegenerative diseases, neurons must be protected against cell death. A number of molecules including anti-tumor agents (cisplatin, adryamicin, β-lap, isothiocyanates, etc.), chemical cancerogens (benzo[*a*]pyrene, etc.), NSAIDs, arsenite, heavy metals, UV, etc might activate the MAP kinase cascades through the ''redox cycling'' by generating oxygen radicals or by inhibiting enzymes involved in ''redox cycling'' such as thioredoxin reductase [48] or quinone reductase [23]. Understanding the effects of pharmacological agents on signaling pathways will help to select the better combination of molecules for the treatment of diseases including cancer and neurodegenerative diseases.

Acknowledgments

This work was also supported by the "Association pour la Recherche contre le Cancer" (grants no. 6624 and 9854 to MP), the CNRS and the University Paris XI. We thank Eric Raze for technical assistance in a part of this work.

References

[1] Murga C, Fukuhara S, Gutkind JS. Novel molecular mediators in the pathway connecting G-protein-coupled receptors to MAP kinase cascades. Trends Endocrinol Metab 1999;10:122–7.
[2] Tournier C, Thomas G, Pierre J, Jacquemin C, Pierre M, Saunier B. Mediation by arachidonic acid metabolites of the H_2O_2-induced stimulation of mitogen activated protein kinase. Eur J Biochem 1997;244:587–95.
[3] Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, Vilcek J. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-jun N-terminal kinase. Proc Natl Acad Sci USA 1997;94:2869–73.
[4] Schwenger P, Alpert D, Skolnik EY, Vilcek J. Cell-type-specific activation of c-jun N-terminal kinase by salicylates. J Cell Physiol 1999;179:109–14.
[5] Giardiello FM. Nonsteroidal anti-inflammatory drugs, eicosanoids, and colorectal cancer prevention. Gastroenterl Clin North Am 1996;25:349–62.
[6] Beazear-Barclay Y, Levy DB, Moser AM, Dove WF, Hamilton SR, Vogelstein B, Kintzler KW. Sulindac supresses tumorigenesis in the Min mouse. Carcinogenesis 1996;17:1757–60.
[7] Boolbol SK, Dannenberg AJ, Chadurn A, Martucci C, Guo X, Ramonetti JT, Abreu-Goris M, Newmark HL, Lipkin ML, DeCosse JJ, Bertagnolli MM. Cyclooxygenase over expression and tumor formation are blocked by Sulindac in a murine model of familial adenomatous polyposis. Cancer Res 1996;56:2556–69.
[8] Jacoby RF, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, Lubet RA, Kellof GJ, Verma A, Moser AR, Dove WF. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal drug piroxicam. Cancer Res 1996;56:710–4.
[9] Thun MJ. NSAID use and decreased risk of gastrointestinal cancers. Gastroenterl Clin North Am 1996;25:333–48.
[10] Oshima M, Dinchu K, Kargman SL, Oshima H, Hancok B, Kwong E, Trzakos JM, Evans JF, Taketo MM. Supression of intestinal polyposis in Apc delta 716 knockout mice by inhibition of cyclooxygenase (cox-2). Cell 1996;87:803–9.
[11] Sheng H, Shao J, Kirkland SC, Tskon P, Coffey R, Morrow J, Beauchamp RD, Dubois RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygense 2. J Clin Invest 1997;99:2254–9.
[12] Kawamori T, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of celocoxib, a specific cyclooxygenase 2 inhibitor, against colon carcinogenesis. Cancer Res 1998;58:409–12.
[13] Kopp E, Ghosh S. Inhibition of NFκB by sodium salycilate and aspirin. Science 1994;265:956–9.
[14] Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T. Salicylates inhibit IκB-α phosphorylation, endothelial-leucocyte adhesion molecule expression and neutrophil transmigration. J Immunol 1996;156:3961–9.
[15] Yamamoto Y, Yin MJ, Lin KM, Gaynor RB. Sulindac inhibits activation of the NFκB pathway. J Biol Chem 1999;274:27307–14.
[16] Dodel RC, Du Y, Bales KR, Paul SM. Sodium salycilate and 17β-oestradiol attenuate nuclear transcription factor NFκB translocation in cultured rat astroglial cultures following exposure to amyloid Aβ(1-40) and lipopolysaccharides. J Neurochem 1999;73:1453–60.
[17] Flynn BL, Theesen KA. Pharmacological management of Alzheimer disease: nonsteroidal antiinflammatory drugs-emerging protective evidence. Ann Pharmacother 1999;33:840–9.
[18] Prasad KN, Hovland AR, Cole WC, Prasad KC, Narheini P, Edwards-Prasad J, Andreatta CP. Multiple antioxidants in the prevention and treatment of Alzheimer disease: analysis of biological rationale. Clin Neuropharmacol 2000;1:2–13.
[19] Kinscherf R, Deigner HP, Haberkorn U. Apoptosis modulators in the therapy of neurodegenerative diseases. Expert Opin Investig Dugs 2000;9:747–64.
[20] Hull M, Lieb K, Fiebich BL. Anti-inflammatory drugs: a hope for Alzheimer disease. Expert Opin Investig Drugs 2000;9:671–83.
[21] Tournier C, Pomerance M, Gavaret JM, Pierre M. MAP kinase cascade in astrocytes. Glia 1994;10:81–8.
[22] Tournier C, Gavaret JM, Jacquemin C, Pierre M, Saunier B. Stimulation of mitogen-activated protein kinase by thyrotropin in astrocytes. Eur J Biochem 1995;228:16–22.
[23] Cross JV, Deak JC, Rich EA, Qian Y, Lewis M, Parrot LA, Mochida K, Gustafson D, Van de Pol S, Templeton DJ. Quinone reductase inhibitors block SAPK/JNK and NFκB pathways and potentiate apoptosis. J Biol Chem 1999;274:31150–4.
[24] Gavaret JM, Matricon C, Pomerance M, Jacquemin C, Toru-Delbauffe D, Pierre M. Activation of s6 kinase in astroglial cells by FGFa and FGFb. Dev Brain Res 1989;45:77–82.
[25] Esfandiari A, Gavaret JM, Lennon AM, Pierre M, Courtin F. Sulfation after deiodination of 3,5,3′-triiodothyronine in rat cultured astrocytes. Endocrinology 1994;135:2086–92.
[26] Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage t4. Nature 1970;227:680–5.
[27] Mc Knight SG. A colorimetric method for the determination of submicrogram quantities of protein. Anal Biochem 1977;78:86–92.
[28] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–54.
[29] Saitoh M, Nishitoh H, Fuji M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK1). Embo J 1998;17:2596–606.
[30] Jaiswal AK, Burnett P, Adesnick M, Mc Bride OW. Nucleotide and deduced aminoacid sequence of a human cDNA (NQO2) corresponding to a second member of NAD(P)H quinone oxidoreductase gene family. Biochemistry 1990;29:1899–906.
[31] Brunmark A, Cadenas E. Redox and adition chemistry of quinoid compounds and its biological implications. Free Rad Biol Med 1989;7:435–77.
[32] Lee SC, Renwick AG. Sulphoxide reduction by rat and rabbit tissues in vitro. Biochem Pharmacol 1995;49:1567–76.
[33] Ichijo H, Nishida E, Irie K, ten Dijke K, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K and Gotoh Y. Induction of apoptosis by ASK1, mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 1997;275:90–4.
[34] Russel J, Spickett CM, Reglinski J, Smith WE, Mc Murray J, Abdullah IB. Alteration of the erythrocyte glutathione redox balance by N-acetyl cysteine, captopril and exogenous glutathione. Febs Lett 1994;347:215–20.
[35] Hosoda S, Nakamura W, Hayashi K. Properties and reaction mechanism of Dt diaphorase from rat liver. J Biol Chem 1974;249:6414–23.
[36] Pink JJ, Planchon SM, Tagliarino C, Varnes ME, Siegel D, Boothman D. NAD(P)H: quinone oxidoreductase activity is the principal determinant of β-laparone cytotoxicity. J Biol Chem 2000;275:5416–24.
[37] Liu H, Nishito H, Ichijo H, Kyriakis JM. Activation of apoptosis signal-regulating kinase 1 by tumor necrosis factor receptor-

associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. Mol Cell Biol 2000;20:2198–208.

[38] Lee SR, Kwon K, Kim SR, Rhee SG. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J Biol Chem 1998;273:15366–72.

[39] Arrigo AP, Landry J, Expression and function of the low-molecular-weight heat shock proteins. In: Morimoto RI, Tissiere A, Georgopoulos C, editors. The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1994. p. 335–73.

[40] Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong AT. p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. J Biol Chem 2000;275:2322–7.

[41] Yu R, Chen C, Mo YY, Hebbar V, Owuor ED, Tan TH, Kong AN. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via Nrf2-dependent mechanism. J Biol Chem 2000;275:39907–13.

[42] Patten EJ, DeLong MJ. Effects of sulindac, sulindac metabolites and aspirin on the activity of detoxification enzymes in HT-29 human colon adenocarcinoma cells. Cancer Lett 2000;147:95–100.

[43] Pearson G, English JE, White MA, Cobb MH. Erk5 and Erk2 cooperate to regulate NFκB and cell transformation. J Biol Chem 2001;276:7927–31.

[44] Moreno-Sanchez R, Bravo C, Vasquez C, Ayala G, Silveira LH, Martinez-Lavin M. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells, and whole heart. Biochem Pharmacol 1999;57:743–52.

[45] Pique M, Barragan M, Dalmau M, Bellosillo B, Pons G, Gil J. Aspirin induces apoptosis through mitochondrial cytochrome c release. FEBS Lett 2000;480:193–6.

[46] Masubuchi Y, Yamada S, Horie T. Diphenylamine as an important structure of nonsteroidal anti-inflammatory drugs to uncouple mitochondrial oxidative phosphorylation. Biochem Pharmacol 1999;58:861–5.

[47] Soh JW, Mao Y, Liu L, Thompson WJ, Pamukcu R, Weinstein IB. Protein kinase G activates the JNK1 pathway via phosphorylation of MEKK1. J Biol Chem 2001;276:16406–10.

[48] Sun QA, Wu Y, Zappacosta F, Jeang KT, Lee BJ, Hatfield DL, Gladyshev VL. Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. J Biol Chem 1999;274:24522–30.