Activation of AP-1 through the MAP Kinase Pathway: A Potential Mechanism of the Carcinogenic Effect of Arenediazonium Ions

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Arenediazonium ions such as those found in the common mushroom *Agaricus bisporus* have been convincingly demonstrated to be tumorigenic. The specific mechanism of their tumorigenicity remains unclear. It has been shown that arenediazonium ions can be metabolized to aryl radicals, and that reaction of these aryl radicals with DNA produces aryl adducts. These metabolic processes also produce the reactive oxygen species superoxide and hydroxyl radicals which have been implicated in AP-1 activation. To further investigate the mechanism of tumorigenesis by arenediazonium ions, we studied the effect of a representative arenediazonium ion on AP-1 activation and phosphorylation of the signal transduction proteins ERK1, ERK2, JNK, and p38 kinase, both in vitro and in vivo. We also identified the specific radicals produced by spin trapping and ESR analysis. Here, it was found that *p*-methylbenzenediazonium ion (**2a**) induced a 16-fold increase in the extent of AP-1 activation at micromolar concentrations, and that this increase coincided with phosphorylation of the signaling kinases ERK1 and -2 and p38 kinase, but not JNK, in JB6 mouse epithelial cells. In vivo studies using AP-1 luciferase reporter-bearing transgenic mice supported the increase in the extent of AP-1 activation in **2a**-treated mice over controls, and showed that this effect was different in different tissue types. The antioxidant *N*-acetylcysteine (NAC), a general antioxidant, showed an inhibitory effect on **2a**-mediated AP-1 induction, while aspirin, a hydroxyl radical scavenger, had no effect. Spin trapping studies showed that while NAC suppressed radical formation from **2a**, aspirin did not alter radical production from **2a**. It appears that **3a**, a carbon-centered radical formed from **2a**, is responsible for AP-1-induced activation, and therefore, radical species that are not oxygen-centered are also capable of inducing AP-1. These results represent a step toward understanding the mechanism of tumorigenicity of arenediazonium ions.

Introduction

The mushroom *Agaricus bisporus* contains arylhydrazines (**1**),[1] arylhydrazides, and arenediazonium ions (**2**) (Figure 1), and many of these compounds are tumorigenic (*1*−*4*). Arenediazonium ions can be metabolically formed from arylhydrazines, azo dyes (*5*−*7*), aryltriazene-based pharmaceutical agents (*8*−*10*), and aryl nitrosamines (*11*). The mechanism by which arenediazonium ions are tumorigenic is unknown. Recent investigations have

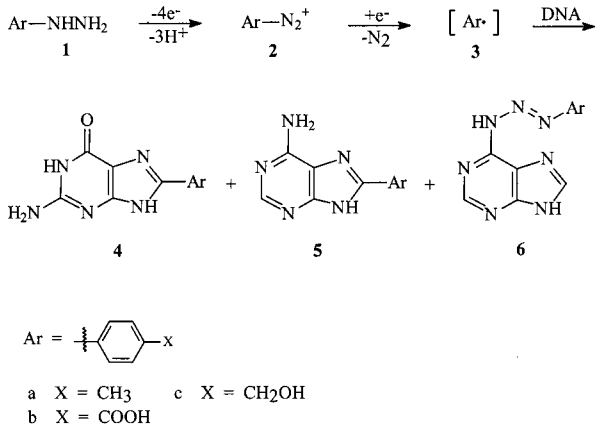

Figure 1. Mechanistic scheme for the metabolic oxidation of arylhydrazines to produce arenediazonium ions, aryl radicals, and DNA C8-arylpurine adducts.

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[1] Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; NAC, *N*-acetylcysteine; PMA, phorbol myristate acetate; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ct DNA, calf thymus DNA; ESR, electron spin resonance; a_N, nitrogen hyperfine coupling constant; a_H, hydrogen hyperfine coupling constant; MEM, modified Eagle's medium; DMPO-OH, 2-hydroxyl-5,5-dimethylpyrrolidine-1-oxyl; DMPO-OOH, 2-hydroperoxyl-5,5-dimethylpyrrolidine-1-oxyl; **1a**, *p*-methylphenylhydrazine; **1b**, *p*-carboxyphenylhydrazine; **1c**, *p*-hydroxymethylphenylhydrazine; **2a**, *p*-methylbenzenediazonium ion; **2b**, *p*-carboxybenzenediazonium ion; **2c**, *p*-hydroxymethylbenzenediazonium ion; **3a**, *p*-methylphenyl radical; **3b**, *p*-carboxyphenyl radical; **3c**, *p*-hydroxymethylphenyl radical; **4a**, C^8-(*p*-methylphenyl)guanine; **4b**, C^8-(*p*-carboxyphenyl)guanine; **4c**, C^8-(*p*-hydroxymethylphenyl)guanine; **5a**, C^8-(*p*-methylphenyl)-adenine; **5b**, C^8-(*p*-carboxyphenyl)adenine; **5c**, C^8-(*p*-hydroxymethylphenyl)adenine; **6a**, N^1-adeninyl-N^3-(*p*-methylphenyl)triazene; **6b**, N^1-adeninyl-N^3-(*p*-carboxyphenyl)triazene; **6c**, N^1-adeninyl-N^3-(*p*-hydroxymethylphenyl)triazene; **7a**, 2-(*p*-methylphenyl)-5,5-dimethylpyrrolidine-1-oxyl.

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suggested that aryl radicals, easily formed by reduction of the arenediazonium ion (*12*−*15*), may be the ultimate tumorigen. The aryl radicals formed by this process can cause DNA damage, including single-strand breaks, double-strand breaks, and DNA cross-links (*15*), and form adducts with purines, their nucleosides, and nucleotides (Figure 1). The products of the reaction of arenediazonium ions with pyrimidines have been studied, though the products of this reaction have not been characterized (*12*, *13*).

Chin et al. (*16*) and Berger et al. (*17*) proposed that the DNA adducts generated from the reaction of purines and arenediazonium ions are the cause of arenediazonium ion tumorigenicity. In their studies, products from both arenediazonium ions and aryl radicals were isolated. Later, Koepke (*11*) suggested that adenosine-derived aryltriazene adducts (**6**) could lead to point mutations in DNA, implicating the arenediazonium ion as the tumorigen. We have suggested that aryl radicals (**3**) derived from arenediazonium ions (**2**) are the ultimate tumorigen and lead to the formation of C-8-arylpurine adducts (Figure 1, **4** and **5**) (*15*, *18*, *19*).

The formation of aryl radicals from arylhydrazines is a two-step process involving (i) oxidative metabolism of arylhydrazines (**1a**−**c**) to arenediazonium ions (**2a**−**c**) (*20*, *21*) and (ii) reduction, with the loss of nitrogen, to aryl radicals (**3a**−**c**, Figure 1) (*15*). We have shown that C^8-arylguanine adducts (**4a**−**c**) are observed in vitro and in cells from **2a**−**c** and that aryl radical formation and C^8-arylguanine adduct formation were correlated (*19*). Aryl radicals appear to be the major radical produced by metabolism of arylhydrazines or arenediazonium ions. However, they are not the only radical species that are produced; both superoxide radical anion and hydroxyl radical may also form. The mechanism of formation of either of these species during arylhydrazine metabolism is complicated, and they may result as byproducts of the metabolism of arylhydrazine to arenediazonium ion. In addition, some arenediazonium ions have been shown to generate hydroxyl radical concomitant with aryl radical production (*15*). Thus, arylhydrazine or arenediazonium ion metabolism will lead to the formation of aryl radicals, superoxide, and hydroxyl radical, and formation of any or all of these radical species may be related to the tumorigenicity of arylhydrazines and arenediazonium ions.

The formation of DNA adducts from arylhydrazines and arenediazonium ions is one possible reason for the tumorigenicity of arylhydrazines. Other mechanisms are possible. For example, AP-1 is an important mediator of tumor promotion involved in a diversity of processes, including cell proliferation, transformation, and apoptosis (*22*−*26*). This factor is a complex protein composed of homodimers or heterodimers of oncogene proteins of the Jun and Fos families. The genes encoding these proteins, *c-jun* and *c-fos*, can be induced by a variety of extracellular stimuli, leading to proliferation. The activity of AP-1 is modulated by several factors, including the redox state of the cell. Evidence suggesting the direct involvement of reactive oxygen species (ROS) in AP-1 activation has been obtained by using defined ROS generating systems to challenge cultured cells.

AP-1 is a transcription factor that interacts with regulatory DNA sequences known as TPA response elements or AP-1 sites (*22*). Many stimuli, including the tumor promoter TPA and ROS, regulate AP-1 binding to the DNA of the promoter region of a number of intermediate genes that govern proliferation and apoptosis (*23*−*25*). It seems plausible that AP-1 activation could occur in response to carbon-centered radicals (alkyl or aryl radicals) in addition to oxygen-centered radicals. AP-1 and its regulated gene expression have been reported to play important roles in neoplastic transformation, tumor progression, and metastasis (*26*−*28*).

On the basis of the importance of AP-1 activity in tumor promotion and progression, we hypothesized that the tumorigenic effect of arylhydrazines and arenediazonium ions could also be mediated through the activation of AP-1 activity. To test this hypothesis, we used JB6 P^+ mouse epidermal cells as an in vitro model. The JB6 family of mouse epidermal clonal genetic variants that are P^+ or P^- provides a suitable model for studying critical gene regulation events that occur during carcinogenesis (*29*). The signal transduction cascades involved in AP-1 activation were also investigated. We demonstrate here that arenediazonium ions are capable of inducing AP-1 activation in in vitro systems and that activation appears to occur through the ERK1 and -2 and p38 kinase signal transduction pathways. The activation of AP-1 is correlated with the formation of aryl radicals from the arenediazonium ions, and both radical formation and AP-1 induction by arenediazonium ions can be inhibited by the addition of *N*-acetylcysteine. Finally, studies using AP-1-luciferase transgenic mice support this AP-1 activation by arenediazonium ions in vivo.

Experimental Procedures

Caution: *Arylhydrazines and arenediazonium ions are tumorigens and should be handled accordingly. Arenediazonium ions, especially when completely dry, are shock sensitive and may detonate.*

Reagents and Plasmids. Arylamines, arylhydrazines, nitrosyl tetrafluoroborate, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), glutathione, and phosphate buffer (100 mM, pH 7.4) were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-Acetylcysteine, aspirin, and Chelex-100 were purchased from Sigma (St. Louis, MO). Ethyl acetate and diethyl ether were purchased from Fisher Scientific (Pittsburgh, PA). The arenediazonium ion *p*-methylbenzene diazonium tetrafluoroborate was prepared as previously described (*30*) from the *p*-toluidine and nitrosyl tetrafluoroborate in ethyl acetate. DMPO was freed of paramagnetic impurities by the method of Floyd (*31*). Water and buffer solutions were treated with Chelex 100 to remove transition ion metal impurities. ESR spectra were measured on a Bruker 300D or Varian E-9 spectrometer in aqueous solutions contained in a quartz flat cell at room temperature. Values for a_N and a_H were obtained by computer simulation using the ESR simulation program PEST (available from http://hippo.niehs.nih.gov/pest1.html).

Eagle's MEM was obtained from Bio Whittaker (Walkersville, MD). Fetal bovine serum, gentamicin, and L-glutamine were purchased from Life Technologies, Inc. (Rockville, MD). Luciferase assay substrate was obtained from Promega (Madison, WI). PhosphoPlus ERK and p38 antibody kits were purchased from New England Biolabs, Inc. (Beverely, MA). JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The AP-1-luciferase reporter plasmid (collagenase-luciferase) was constructed as reported previously (*32*, *33*). PD98059 and SB203580, which are specific MEK1 (upstream of ERK) and p38 kinase inhibitors (*34*, *35*), respectively, were from Calbiochem (San Diego, CA).

Cell Culture. The $JB6^+$ mouse epidermal cell line, which was stably transfected with the AP-1-luciferase reporter plasmid and NfκB-CAT reporter plasmid (JB6/AP/κB) (*36*), was cultured

in Eagle's MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 50 μg/mL gentamicin. The cells were grown at 37 °C in a 5% CO_2 medium for the experiments.

Assay of AP-1 Activity in Vitro. A confluent monolayer of JB6/AP/κB cells was trypsinized, and 5×10^4 viable cells (suspended in 1 mL of Eagle's MEM supplemented with 5% FBS) were added to each well of a 24-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Twelve hours later, cells were cultured in Eagle's MEM supplemented with 0.5% fetal bovine serum for 12−24 h to minimize basal AP-1 activity and then exposed to **2a** in the same medium to test the effects on AP-1 induction. The cells were lysed with 200 μL of 1× lysis buffer provided in the luciferase assay kit by the manufacturer, and the luciferase activity was measured. The results, obtained using an Analytical Luminescence luminometer (Sparks, MD), are expressed as relative AP-1 activity counts compared with untreated controls.

Animals and Administration of 2a. Transgenic mice that carry and express the AP-1-luciferase reporter (2× TPA response element-binding sites) were originally established by Rincon and Flavell (*37*). A C57BL/6 male mouse carrying the 2× TPA response element-luciferase transgene was crossed with a DBA2 female (SASCO, Omaha, NE) (*38*). The offspring were screened for the presence of luciferase activity. Males and females were housed separately in solid-bottom polycarbonate cages on ventilated animal racks (four to five mice per cage) under temperature-, humidity-, and yellow light-controlled conditions. Food and water were available ad libitum.

The AP-1-luciferase reporter-bearing male and female mice (8−12 weeks old) were randomly divided into six groups consisting of eight mice in each group. **2a** (100−300 mg/mouse) was instilled by gavage in water. Control mice were gavaged with saline.

Assay of AP-1 Activity in Vivo. One to two days after the administration of **2a**, the mice were sacrificed by exsanguination under deep pentobarbital anesthesia. Tissue samples were removed from the lung, liver, kidney, stomach, and spleen and minced with scissors. Lysis buffer (100 μL/10 mg of tissue) was added, and the tissues were lysed overnight at 4 °C. The luciferase activity of the tissue supernatant obtained after lysis was measured with a luminometer as described previously (*38*). AP-1 activity is expressed relative to the level of luciferase activity of the controls.

Protein Kinase Phosphorylation Assay. Immunoblotting for phosphorylation of ERKs and p38 kinase was carried out as described by the protocol of New England Biolabs, Inc., and immunoblotting for phosphorylation of JNKs was carried out as described by the protocol of Santa Cruz Biotechnology, using phosphospecific antibodies against phosphorylated sites of ERKs, p38 kinase, and JNKs. Non-phospho-specific antibodies against ERKs, JNKs, and p38 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay using the same transferred membrane blot.

ESR Spin-Trapping Studies of 2a. ESR studies were conducted with freshly prepared solutions of **2a** (6 mM), DMPO (60 mM) in water, NAC (6 mM), and aspirin (6 mM) in phosphate buffer (100 mM, pH 7.4). All solutions were treated with Chelex-100 to remove transition metals. The final concentrations, unless otherwise noted, were **2a** (2 mM), DMPO (20 mM) in water, NAC (2 mM), and aspirin (2 mM). Solutions were mixed in a separate test tube and transferred to the flat cell and spectral measurements initiated within 1 min of mixing. Spectrometer parameters were as follows: spectral width, 100 G; microwave power, 20 mW; time constant, 0.04 s; modulation amplitude, 0.5 G; and receiver gain, 2×10^4.

Statistical Analysis. The data are the means ± the SE of values compared and analyzed using a one-way analysis of variance. The statistical significance was determined by a two-tailed Student's t test for paired data and is considered significant at $p \leq 0.05$.

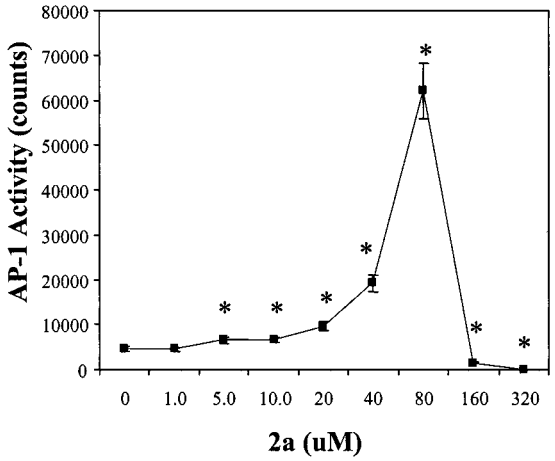


Figure 2. Arenediazonium ion **2a**-induced AP-1 activation in JB6 P^+ cells. JB6/AP-1/κB cells (5×10^4 cells in 1 mL of MEM with 5% fetal bovine serum) were seeded into each well of a 24-well plate, cultured overnight at 37 °C, and then cultured in MEM and 0.5% fetal bovine serum for 12 h. The cells were then treated with **2a** at the indicated concentration in the same medium for 24 h. AP-1 activity was measured by the luciferase activity assay as described in Experimental Procedures. The results, presented as reporter activities, are means ± the SE of three assay wells from three independent experiments. Values denoted with asterisks are statistically different from the control value.

Table 1. Effect of Inhibitors on 2a Activation of AP-1[a]

conditions[b]	AP-1 activity ($\times 10^{-3}$)[c]	conditions[b]	AP-1 activity ($\times 10^{-3}$)[c]
control	4.61 ± 0.53	**2a** and NAC	6.33 ± 0.44*
PMA	17.6 ± 1.09*	aspirin	1.08 ± 0.24*
2a	19.5 ± 1.11*	**2a** and aspirin	20.6 ± 1.20*
NAC	3.04 ± 0.29*		

[a] The general conditions that were used are described in the legend of Figure 2. [b] The following concentrations were used: 40 μM **2a**, 20 μg/mL PMA, 40 μM NAC, 40 μM aspirin. [c] The AP-1 activities are presented as reporter activities and represent the results of six measurements. Errors are reported ±SE. Values with asterisks are statistically different from the control value.

Results

Arenediazonium Ion 2a Causes AP-1 Activation in JB6 Cells. To explore the effects of **2a** on the induction of AP-1 activity, 5×10^4 JB6/AP/κB cells were exposed to varying doses (1−320 μM) of **2a** (Figure 2). The AP-1 activation attained significance at a low concentrations of **2a** (10−20 μM) and reached a maximum at approximately 80 μM. Above this concentration, the apparent extent of AP-1 activation decreased due to cell death. On the basis of this result, 40 μM was used as the concentration for time course studies. At intervals from 12 to 72 h, the relative AP-1 activity was tested using the luciferase assay, and a maximum response was observed at approximately 24 h.

Effect of Radical Inhibitors on AP-1 Activation in JB6 Cells. The *p*-methylbenzenediazonium ion **2a** has been shown to produce aryl radicals in cells (*39*) in vitro and in cells. For investigation of the role that aryl radicals may play in the activation of AP-1, JB6 cells were first preincubated with the radical inhibitor NAC or aspirin and then treated with **2a**. In the absence of **2a**, both NAC and aspirin reduced the extent of AP-1 activation (Table 1). The combination of NAC and **2a** resulted in a significant decrease in the extent of AP-1 activation relative to JB6 cells treated with **2a** alone.

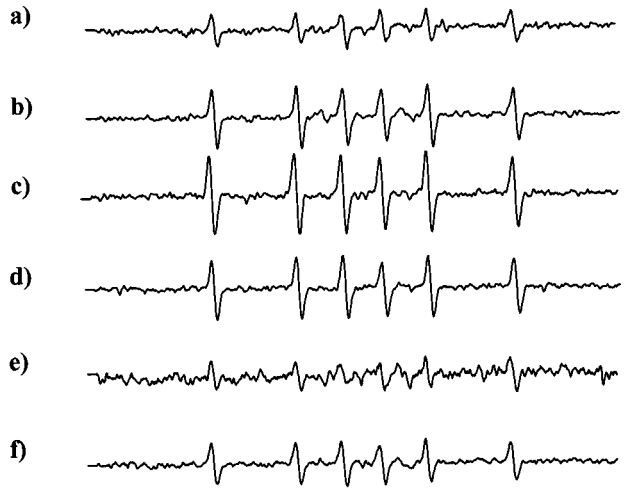
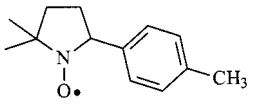


Figure 3. ESR spectra of **2a** with and without NAC or aspirin. All spectra were recorded on samples dissolved in phosphate buffer (100 mM, pH 7.4) and DMPO (20 mM) and (a) **2a** (2.0 mM) immediately after mixing, (b) **2a** (2.0 mM) after 15 min, (c) **2a** (2.0 mM) added to NAC (2.0 mM) immediately after mixing, (d) **2a** (2.0 mM) added to NAC (2.0 mM) after 15 min, (e) **2a** (2.0 mM) added to aspirin (2.0 mM) immediately after mixing, and (f) **2a** (2.0 mM) added to aspirin (2.0 mM) after 15 min. In all cases, the concentrations refer to the final concentration. All spectra were recorded under identical spectrometer settings.

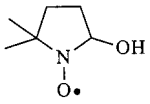
However, aspirin had no effect on the level of activation induced by JB6 cells treated with **2a**.

Effect of Radical Inhibitors on Spin Adduct Formation. When **2a** was added to phosphate buffer containing DMPO, a six-line ESR spectrum was observed corresponding to the *p*-methylphenyl-DMPO adduct (**7a**) (Figure 3a).



7a

The magnitude of this signal gradually increased with time, and after approximately 15 min, an additional though very weak four-line spectrum could also be discerned, which corresponds to the hydroxyl radical−DMPO adduct (DMPO-OH) (Figure 3b).



DMPO-OH

The addition of a small amount of NAC to **2a** initially resulted in an initial burst in the amount of the **7a** that formed (Figure 3c), but the magnitude of this signal began to quickly decrease (Figure 3d). In addition, formation of DMPO-OH was not observed under these conditions (Figure 3). A similar result was obtained if **2a** was added to a solution containing an excess of NAC (data not shown).

When these same experiments are performed with aspirin replacing NAC, a significantly different behavior was observed (Figure 3e,f). When **2a** was added to aspirin, the radical **7a** was observed (Figure 3e) and the magnitude of this signal grew over time (Figure 3f). In addition, in the latter spectrum (t = 15 min), some

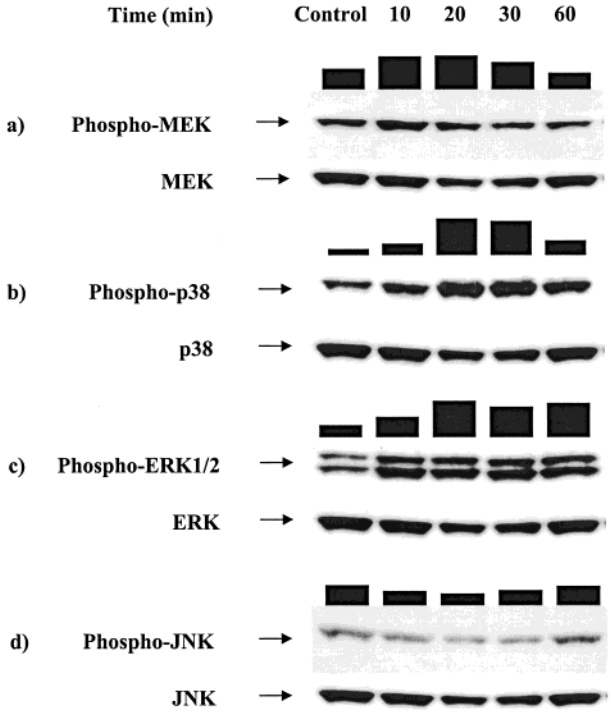


Figure 4. Arenediazonium ion **2a** stimulated the phosphorylation of p38, MEK, ERK1 and -2, and JN MAPK. JB6 P^+ cells were cultured in MEM containing 5% FBS in six-well (35 mm diameter) plates until 80% confluent and then cultured in MEM containing 0.5% FBS for 24 h. After this time, the cells were exposed to **2a** (40 mM) dissolved in the same medium for different times as indicated. The cells were lysed, and phosphorylated and nonphosphorylated (a) MEK kinase proteins were assayed using the PhosphoPlus MAPK kit (0.25−0.41), (b) p38 kinase proteins were assayed using the PhosphoPlus MAPK kit (0.52−3.12), (c) ERK1 and ERK2 proteins were assayed using the PhosphoPlus MAPK kit (2.86−8.71), and (d) phospho-specific JNK antibody and regular JNK antibody (0.14−0.24) were assayed. The phosphorylated and nonphosphorylated proteins were analyzed using the same transferred membrane blot. The bars above each gel were prepared from densitometry data. Due to the range of densities, vertical scales differ and are in parentheses for each plot (minimum minus maximum).

DMPO-OH was also observed, though the signal was weak. This behavior was nearly identical with that observed for **2a** to which neither NAC or aspirin had been added (compare panels a and e of Figure 3 and panels b and f of Figure 3).

Activation of ERKs and p38 Kinase by 2a in JB6 Cells. Since mitogen-activated protein kinases, including p38 kinase, ERKs, and JNKs, are the upstream kinases responsible for c-Jun phosphorylation and AP-1 activation (*40*−*43*), we tested which class of MAPK was involved in the AP-1 activation by **2a**. We examined the influence of **2a** on the phosphorylation of ERK1 and -2, JNKs, and p38 kinases. Using antibodies specific for the above MAPK family and phospho-specific for the phosphorylated MAPKs, we studied ERK1 and -2, JNKs, and p38 kinase proteins and the protein phosphorylation of ERK1 and -2, JNKs, and p38 kinase in JB6 P^+ cells. Exposure of JB6 P^+ cells to **2a** stimulated the phosphorylation of p38 kinase and ERKs but not JNKs. The time course of p38 kinase phosphorylation induced by **2a** (40 μM) is shown in Figure 4b. Phosphorylation of p38 kinase was apparent within 10 min, and maximal activation was observed 30 min after exposure. Interestingly, when phosphorylation of MEK3/6 was probed, which is upstream of p38, maximum phosphorylation was reached

Table 2. Inhibition of 2a-Induced AP-1 Activation by PD98059 and SB203580[a]

[SB203580] (μM) (p38 inhibitor)	AP-1 activity[b] ($\times 10^{-3}$)	[PD98059] (μM) (ERK inhibitor)	AP-1 activity[b] ($\times 10^{-3}$)
0	19.9 ± 4.0	0	19.8 ± 3.9
10	15.5 ± 3.1	2.5	19.0 ± 3.7
20	12.5 ± 2.5*	5	15.7 ± 3.1
40	10.2 ± 2.1*	10	10.0 ± 2.1*
80	7.6 ± 1.5*	20	1.0 ± 0.2*

[a] JB6 P^+ cells (5×10^4) were seeded into each well of a 24-well plate. After being cultured overnight at 37 °C, the cells were cultured in MEM containing 0.5% FBS for 12 h. The cells were then pretreated with various concentrations of the p38 inhibitor SB203580 or the ERK inhibitor PD98059 for 1 h and then exposed to **2a** (40 μM) in the presence of the inhibitors for 24 h. [b] AP-1 activity was measured by the luciferase activity assay as described in Experimental Procedures. The results, presented as luciferase activities, are means ± the SE of three assay wells from three independent experiments. Values with asterisks are statistically different from the control value. Proteins were analyzed using the same transferred membrane blot.

between 10 and 20 min and decreased thereafter (Figure 4a). **2a** also induced phosphorylation of ERK1 and -2. A time dependence similar to that observed for p38 was seen, rising to a maximum around 30 min (Figure 4c). In contrast, **2a** did not activate phosphorylation levels of JNKs and may actually be slightly inhibitory (Figure 4d). We note that in the study presented here only one JNK isoform was detected rather than two, the typical situation. However, we have observed this in other systems, and the phenomenon may be related to the source of the antibody (*44*).

Inhibition of ERK1 and -2 or p38 Kinases by Specific Inhibitors Also Blocks 2a-Induced AP-1 Activation. To further confirm that activation of AP-1 by **2a** is mediated through p38- and ERK-dependent signal transduction pathways, we examined the effects of PD98059 and SB203580 on **2a**-induced activation. PD98059 has been shown to act as a highly selective inhibitor of MEK1 and -2 activation, whereas SB203580 has been shown to be a specific inhibitor of p38 kinase. MEK1 and -2 is an upstream activator (kinase) of ERK1 and -2. **2a**-induced AP-1 activation was significantly inhibited by 10 μM PD98059 or 20 μM SB203580 (Table 2).

Transactivation of AP-1 by 2a in AP-1-Luciferase Reporter Transgenic Mice. To determine whether similar mechanisms exist in vivo, we used AP-1-luciferase reporter transgenic mice for these studies. The transgenic mice were exposed to **2a** by gastric gavage. At 1−2 days after exposure, animals were anesthetized with sodium pentobarbital and sacrificed by exsanguination; tissues from the lung, liver, kidney, stomach, and spleen were removed, and their luciferase activities were measured as described in Experimental Procedures. Elevated levels of AP-1 transactivation were detected in stomach and kidney tissue, as shown in Table 3.

Table 3. Activation of AP-1 in Vivo in AP-1-Luciferase Reporter Transgenic Mice[a]

tissue	control	experimental
stomach	165 ± 24	12500 ± 1300*
lung	112 ± 17	154 ± 23
kidney	267 ± 40	20000 ± 2000*
spleen	54 ± 8	72 ± 11
heart	101 ± 15	134 ± 20
liver	132 ± 20	162 ± 24

[a] AP-1 activity was measured by the luciferase activity assay as described in Experimental Procedures. The results, presented as luciferase activities, are means ± the SE of three assays from two independent experiments. Values with asterisks are statistically different from the control value.

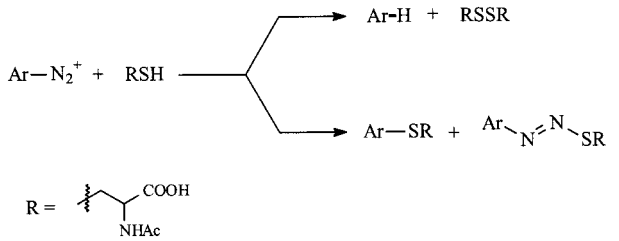


Figure 5. Reactions of NAC with arenediazonium ions **2** and aryl radicals **3**.

Discussion

Human exposure to arylhydrazines, arenediazonium ions, and precursors to arenediazonium ions such as azo dyes (*5*−*7*), aryltriazene-based pharmaceutical agents (*8*−*10*), and aryl nitrosamines (*11*) is likely and may pose risks due to the tumorigenic nature of arenediazonium ions (*1*−*4*). The mechanism whereby arenediazonium ions are tumorigenic is unknown. We have previously shown that arenediazonium ions and derived aryl radicals can produce DNA damage (*15*) and DNA adducts (*18*, *19*). We have also shown that arenediazonium ions are tumorigens in mice (*5*, *45*, *46*). Furthermore, since the formation of radicals and aryl−DNA adducts correlates with tumor formation, we have proposed that DNA adduct formation is related to the tumorigenic nature of the arenediazonium ions. Here, we have explored an additional mechanism of tumorigenesis by arenediazonium ions, that of AP-1 induction. In particular, activation of specific MAP kinases involved in AP-1 induction by arenediazonium ions such as **2a** appears to be a primary event in the initiation of cascades at the cell membrane level, leading to signal transduction and the induction of early response genes that may be critical in carcinogenesis.

The arenediazonium ion **2a** was found to activate AP-1 in a dose-dependent fashion. While only minor induction was seen at concentrations in the range of 0−20 μM, an up to 16-fold increase in JB6 cells was seen in the concentration range of 20−80 μM. Further increases in arenediazonium ion concentration (80−320 μM) were found to be cytotoxic (Figure 2).

The induction of AP-1 by **2a** could be modulated by the addition of NAC. NAC is a known inhibitor of radicals by reduction. However, NAC is also nucleophilic, and therefore, there are two possible mechanisms for inhibition of **2a** AP-1 induction by NAC. One mechanism simply involves hydrogen abstraction from the thiol group of NAC, resulting in the formation of toluene and the disulfide of NAC. Alternatively, NAC may add to the arenediazonium ion to yield an aryl thiodiazotate or, more likely, a thioether as shown in Figure 5 (*47*).

It may be noted aspirin did not prevent AP-1 induction by **2a** and did not significantly reduce the total amount of DMPO-trapped radical. The reaction of either hydroxyl or aryl radicals with NAC is a reduction and only requires the transfer of an electron which should be fairly rapid for either type of radical. In contrast, the reaction mechanism whereby aspirin destroys radicals is not a reduction, but rather, radicals either abstract a hydrogen atom (*48*) or are trapped by covalent bond formation (*49*,

50). For the more reactive hydroxyl radical, reaction with aspirin is rapid and competitive with the trapping reaction with DMPO.

The ESR data support the involvement of aryl radicals as the cause of AP-1 induction. In the absence of radical inhibitors, a signal corresponding to the spin adduct **7a** was observed to slowly grow in (Figure 3a), along with what appears to be a small amount of DMPO-OH (Figure 3b). Addition of **2a** to a solution of DMPO and NAC produces a large amount of spin adduct **7a** (Figure 3c) which then gradually decreases with time (Figure 3d). In contrast, aspirin has little effect on the formation of **7a**. The spectra obtained in the presence or absence of aspirin were nearly the same with respect to the quantity and time course of formation of **7a** (Figure 3e,f).

These observations correlate with the trends observed for AP-1 induction by **2a** alone and the effects of added NAC or aspirin. In the absence of a radical inhibitor, the diazonium ion is slowly reduced to the aryl radical **3a**, which in turn is trapped by DMPO to give **7a**. In contrast, when NAC is present, the arenediazonium ion **2a** is rapidly reduced and either trapped by DMPO or further reduced to toluene. After the initial burst of **7a** formation, no more is formed and the DMPO adduct **7a** slowly decomposes. Note that if the initial concentration of NAC is much greater than that of **2a** (20 times), then very little **7a** is formed since the reduction of the aryl radical **3a** by NAC becomes the dominant process (data not shown). Finally, since the diazonium ion is not reactive toward aspirin, it has no effect on the arenediazonium ion concentration and the ESR spectra resemble those obtained in the absence of aspirin. This can be seen by comparison of the spectra in panels a and e and panels b and f of Figure 3. The lack of any observable effect by aspirin, with respect to either AP-1 induction or the ESR spectra of **2a** in the presence of aspirin, suggests that it is the aryl radical **3a** that is inducing AP-1 and not hydroxyl radical.

The ESR studies are consistent with aryl radical induction of AP-1 based on the correlation observed between the AP-1 inhibition data and the ESR data (Figures 2 and 3). However, we have no direct evidence that the effects of NAC and aspirin in phosphate buffer and in cells are the same. In previous studies, we have found by ESR that the aryl radical and hydroxyl radical formation in simple buffer or buffer- and reductant-containing solutions and biological systems (microsomes or cells) are similar (*39*). Similar findings have been obtained with other carcinogenic compounds such as Cr(VI), which produce radical species and damage DNA (*51*). These studies have shown that similar ESR spectra are observed in the presence of NAC or aspirin or without any added reducing agent under cell free conditions and in the presence of cells. In addition, we have shown that radical formation from Cr(VI) occurs in cells (*51*). Thus, it is likely that the ESR spectra, obtained here from **2a** or **2a** and a reductant, but in the absence of JB6 cells, are indicative of radical formation in JB6 cells.

It has been shown in a variety of systems that AP-1 plays an important role in the preneoplastic-to-neoplastic transformation in cell culture (*24*). AP-1 is an important mediator of tumor promotion and is involved in many processes. This transcription factor can alter gene expression in response to many stimuli, including the tumor promoter TPA, epidermal growth factor (EGF), and tumor necrosis factor α (TNF-α) (*24*). Some of the genes

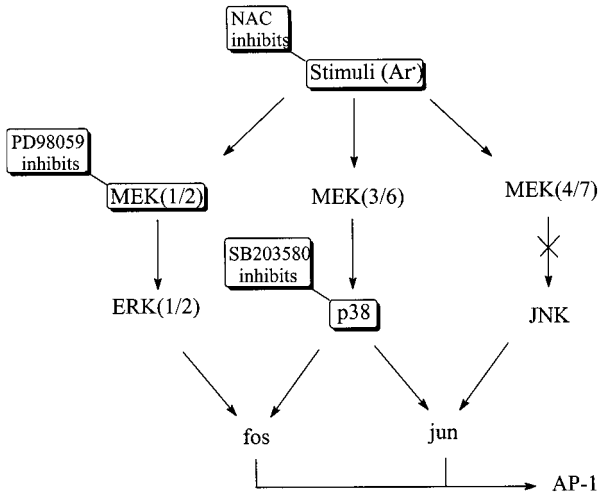


Figure 6. Simplified MAPK pathway cartoon. The figure indicates the pathways induced by arenediazonium ions (ERK and p38) and points of inhibition by NAC, the p38-specific inhibitor SB203580, and ERK-specific inhibitor PD98059.

regulated by AP-1 are involved in tumor promotion and progression such as the cytokines interleukin-1, TNF-α, collagenase V, and stromelysin (*52*−*54*). Overexpression of *c-jun* in JB6 cells causes neoplastic transformation and inhibition of AP-1 activity by fluocinolone acetonide or molecular biological inhibitors such as dominant-negative *c-jun* and initiates cell transformation (*27*, *33*, *36*, *55*−*57*).

The signal transduction pathways leading to transcription factor activation have been extensively studied. It is believed that stress-related signals, such as UV light or radical species such as ROS, can induce the activation of MAPK pathways, including ERKs, JNKs, and p38 (Figure 6). AP-1 is the downstream target of these three pathways. In this study, we have examined the possible role of each MAPK family member in **2a**-induced AP-1 activation. We have found that **2a** induced phosphorylation of ERKs and p38 kinase (Figure 4b,c). Induction of the MEK3/6, which is upstream of p38, was also induced and with a time course that demonstrated that it is induced earlier than p38 (Figure 4a,b). JNK was not found to be induced in these studies, and the data suggest that **2a** may even slightly inhibit JNK (Figure 4d).

Pretreatment of the JB6 cells with the p38 inhibitor SB203580 inhibited AP-1 transactivation. The inhibitor SB203580 has been studied extensively and has generally been shown to be a selective inhibitor of p38 and does not inhibit MEK, ERK, or JNK (*35*, *58*, *59*). Activation of ERK was also observed, and the role of ERK in activation of AP-1 is confirmed with the ERK-specific inhibitor PD98059. PD98059 has been shown to be selective for inhibiting phosphorylation of MEK1 and -2 to ERK1 and -2. Thus, these results suggest that **2a**-induced AP-1 activation is only through p38 and ERK1 and -2 pathways.

The development of AP-1-luciferase transgenic mice makes it possible to study the role of AP-1 activtion in tumor promotion in vivo (*60*). The results obtained in this study show that **2a** is able to cause AP-1 activation in transgenic mice. Maximal activation occurred at a dose of 100 mg/mouse for 2 days and was organ-type-dependent (activation observed in stomach and kidney tissue). However, the cell types involved in this AP-1 activation have not yet been identified. Additional studies are

required to answer this question. It may be noted that previous studies have shown that arenediazonium ions administered by gavage, including **2a**, produce stomach tumors (*61*). In addition, studies wherein the mushrooms were fed to mice also showed that they produced stomach tumors (*62*). In neither study, however, were kidney tumors observed.

In summary, using the AP-1-luciferase reporter cell model, we have demonstrated that **2a** induces AP-1 activation. This induction is through the MAPK p38 and ERK pathways. Arenediazonium ion-induced activation of AP-1, which appears to be mediated by aryl radicals, may be involved in the tumorigenicity of **2a** and of arenediazonium ions in general. Finally, the activation of AP-1 appears to be more related to aryl radicals derived from arenediazonium ions rather than to the arenediazonium ion itself.

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