Mitogen-Activated Protein Kinase Activation by Oxidative and Bacterial Stress in an Amphibian Cell Culture Model

Lisa A. Carter,1,2 Maija B. Tabor,1,2 James C. Bonner,2 and Lisa A. Bonner1

1Peace College, Raleigh, North Carolina, USA; 2National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

The decline of many amphibian species could be caused by their susceptibility to environmental pollutants that cause cellular stress and cell death. A variety of intracellular signal transduction pathways are activated by environmental stress factors, which result in cell death. Mitogen-activated protein kinases are intracellular signaling molecules that include the extracellular signal-regulated kinases (ERK-1 and ERK-2). We used cultured Xenopus tadpole cells (XTC-2 cells) to investigate the activation of ERK by oxidative or bacterial stress, two environmental factors that could contribute to pollution in aquatic systems. We exposed XTC-2 cell monolayers to hydrogen peroxide or bacterial lipopolysaccharide and measured ERK activation by Western blotting using antibodies raised against phosphorylated ERK-1 and ERK-2. Only ERK-2 was detected in XTC-2 cells. Both hydrogen peroxide and lipopolysaccharide caused ERK-2 phosphorylation in a time- and concentration-dependent manner. Hydrogen peroxide caused a 20- to 30-fold increase in ERK-2 activation that peaked 30 min after treatment, and lipopolysaccharide induced a 5- to 10-fold increase in ERK-2 activation that peaked 60 min after treatment. PD98059, an inhibitor of the ERK pathway, reduced the cytotoxic response of XTC-2 cells to hydrogen peroxide or lipopolysaccharide. These data suggest that ERK-2 is an intracellular target of oxidative and bacterial stress in amphibians that mediate, at least in part, the cytotoxic response to hydrogen peroxide or lipopolysaccharide. Moreover, the Xenopus (XTC-2) cell culture system could serve as a useful model to identify agents that might threaten amphibian populations and human health.

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Amphibians are particularly sensitive to environmental stress due to their unique life-history strategies that lead to their exposure to natural and anthropogenic perturbations in both aquatic and terrestrial ecosystems. Because amphibians have thin, permeable skin and their eggs lack shells, they are susceptible to environmental exposure during development and as adults. Therefore, they may serve as important sentinel species to changes in environmental conditions. Since the mid-1990s, unusually large numbers of frogs with malformations have been found in North America (1,2). Abnormalities have been reported in 36 species of amphibians from 42 U.S. states. There is also concern that the environmental factors causing these anuran deformities may affect human health. The reason for the amphibian malformations and population decline remains unknown, although it has been suggested that parasitic infections, UV radiation, and chemical pollutants could play a role (3). If endoparasitic infestations are involved, it is likely that environmental stress (i.e., UV radiation, pollution) could increase susceptibility to these infections (4). It is also reasonable to assume that a number of critical environmental interactions could contribute to the detrimental effects on amphibian populations (5,6).

There has also been a rapid decline and range reduction of certain amphibian species worldwide over the past two decades. However, some species within the same regions do not appear to be affected, whereas others are driven to extinction (7). Although the explanation for the degradation of amphibian populations is currently disputed, Pechmann et al. (8) caution that it may be difficult to distinguish natural population fluctuations from human-induced declines without long-term surveys at numerous sites to isolate the various confounding components. However, anthropogenic factors such as water pollution and atmospheric conditions such as ozone depletion and/or UV radiation could play important roles (4,6). Continuing population reductions and morphologic defects in anurans has generated international interest. A primary consideration in the correlation of this problem to human health is our dependence on water quality. Therefore, frogs are an important sentinel species that may indicate environmental deterioration. Although there has been much investigation into the causal factors contributing to malformations and declining amphibian numbers, in vitro cell culture models to address which environmental pollutants cause cellular stress in amphibians are lacking.

A major indicator of cellular stress is the activation of a class of intracellular proteins called mitogen-activated protein (MAP) kinases, which are a family of serine/threonine kinases that include extracellular signal-regulated kinases (ERKs), p38 MAP kinases, and c-jun N-terminal kinases (JNKs). MAP kinases normally regulate a variety of cellular activities in response to endogenous cytokines and growth factors, leading to biologic outcomes such as cell growth and differentiation (10). Several environmental agents activate MAP kinases to cause cellular stress and cytotoxic responses, including bacterial products (such as lipopolysaccharide; LPS), pesticides, certain transition metals, and UV radiation (11–15). Transition metals and UV radiation cause MAP kinase activation via the generation of reactive oxygen species (14,15). In particular, UV radiation has been implicated as a major factor in amphibian mortality and deformities (16–19).

In this study, we investigated MAP kinase (ERK) activation in Xenopus tadpole cells (XTC-2). The XTC-2 cells were treated with a reactive oxygen species (hydrogen peroxide; H2O2) or a bacterial wall product, LPS. Both of these factors are associated with environmental stress in polluted aquatic ecosystems (20,21). H2O2 and LPS both caused phosphorylation of ERK-2 in XTC-2 cells in a time- and dose-dependent manner. These data suggest that the ERK-2 pathway is a sensitive biomarker to detect environmental stress and that the ERK pathway mediates cell death in response to H2O2 and LPS.

Materials and Methods

Materials. Goat polyclonal anti-ERK-1/2 (catalog no. SC-154-G) that recognizes total cellular ERK (i.e., both phosphorylated and unphosphorylated forms) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). This antibody was raised against an epitope mapping the carboxy terminus of MAP kinases normally regulate a variety of cellular activities in response to endogenous cytokines and growth factors, leading to biologic outcomes such as cell growth and differentiation (10). Several environmental agents activate MAP kinases to cause cellular stress and cytotoxic responses, including bacterial products (such as lipopolysaccharide; LPS), pesticides, certain transition metals, and UV radiation (11–15). Transition metals and UV radiation cause MAP kinase activation via the generation of reactive oxygen species (14,15). In particular, UV radiation has been implicated as a major factor in amphibian mortality and deformities (16–19).

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Address correspondence to L. A. Bonner, Pressly Building, Peace College, 15 East Peace Street, Raleigh, NC 27604 USA. Telephone: (919) 508-2266. Fax: (919) 508-2326. E-mail: bonnerl@peace.edu

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ERK-2-encoded MAP kinase p42 of rat origin. The antibody is reactive with ERK-2 and to a lesser extent ERK-1 and possesses broad interspecies reactivity (murine, human, chicken, and frog). To verify that we were detecting only ERK-2 and not ERK-1 in XTC-2 cells, we used two other antibodies raised against total ERK-1 and -2 from Transduction Laboratories (Lexington, KY) or Upstate Biotechnologies (Lake Placid, NY). An anti-phospho-ERK-1/2 monoclonal antibody that is specific for the phosphorylated forms of ERK-1/2 (catalog no. 9106) was purchased from Cell Signaling (Beverly, MA). This antibody had previously been reported by the company to cross-react with ERK-1/2 from human, rat, mouse, and hamster (no information on Xenopus was available). Anti-mouse IgG-HRP was purchased from DakoPatts (Carpenteria, CA). 

We investigated Xenopus tadpole cells (XTC-2) as an in vitro model of oxidant- and bacterial-induced cellular stress. We first determined whether XTC-2 cells possessed either the p42 (ERK-2) or p44 (ERK-1) isozymes of MAP kinase.

**Results**

XTC-2 cells contain ERK-2. We investigated MAP kinase activation in confluent, quiescent cultures of Xenopus (XTC-2) cells as an in vitro model of oxidant- and bacterial-induced cellular stress. We first determined whether XTC-2 cells possessed either the p42 (ERK-2) or p44 (ERK-1) isozymes of MAP kinase.

![Figure 1](image1.png)  Expression of the MAP kinase ERK-2 in amphibian cells (XTC-2) and in human lung fibroblasts (HLF). Cell lysates from confluent, quiescent cultures of XTC-2 cells and HLF cells were assayed by Western blotting using an anti-ERK antibody that detects unphosphorylated ERK-1 and ERK-2. XTC-2 cells contained only ERK-2 whereas both ERK-1 and ERK-2 were present in HLF cells.

![Figure 2](image2.png)  Western blot analysis showing phosphorylation of ERK-2 by H2O2 in a time-dependent manner. Confluent, quiescent XTC-2 cells were treated with H2O2 (10 µM) for 15, 30, 60, and 120 min. (A) Representative Western blot experiment showing that maximum phosphorylation of ERK-2 was observed at 30 min after treatment with H2O2. (B) Relative levels of ERK-2 activation determined by densitometric scanning of the phospho-ERK bands and normalized to the ERK signal. Data are the means ± SEM of three separate experiments and are presented as the phospho-ERK/ERK ratio. One-way ANOVA yielded a calculated F value of 3.12 (p = 0.049).
We detected only ERK-2 (p42 MAP kinase) in XTC-2 cells, whereas both ERK-1 and ERK-2 were readily detectable in human lung fibroblasts (Figure 1). Similar results were obtained using two other polyclonal antibodies raised against total ERK-1 and ERK-2 (data not shown). Therefore, although we cannot rule out that XTC-2 cells might possess ERK-1, we clearly demonstrated that our assay detects only ERK-2.

**Rapid activation of ERK-2 by oxidative stress.** We next evaluated whether oxidative stress would activate ERK-2 in XTC-2 cells, as has been reported in mammalian cell culture systems (23). ERK-2 phosphorylation peaked 30 min after H2O2 treatment, causing a 25-fold increase in the phospho-ERK-2/ERK-2 ratio, and gradually declined over 2 hr. One-way ANOVA for the H2O2 time course yielded a calculated F value of 3.12 (p = 0.049); Figure 2). The 30-min time point was used for further experiments evaluating the concentration-dependent activation of ERK-2. H2O2-induced ERK-2 activation was maximal at 10 µM H2O2 and did not significantly increase further at 50 µM H2O2 (Figure 3). One-way ANOVA for the H2O2 dose response yielded a calculated F value of 3.85 (p = 0.038). In all of these experiments, the signal for total ERK-2 protein did not change with H2O2 treatment.

**Delayed activation of ERK-2 by LPS.** We then investigated whether ERK-2 in XTC-2 cells was a target for endotoxin-mediated cell stress. ERK-2 phosphorylation peaked 60 min after LPS treatment, causing a 5- to 10-fold increase in the phospho-ERK-2/ERK-2 ratio (Figure 4). These data indicated that LPS was a weaker activator of ERK-2 compared to H2O2, and the ERK-2 phosphorylation response to LPS was delayed by as much as 30 min compared to H2O2. LPS caused a concentration-dependent increase in ERK-2 activation that was maximal at 50–500 µg/mL LPS (Figure 5). One-way ANOVA yielded a calculated F value of 2.57 (p = 0.038) for the LPS time-course data (Figure 4) and of 2.80 (p = 0.08) for dose response (Figure 5).

**Effect of ERK inhibition on H2O2- and LPS-induced cytotoxicity.** H2O2 and LPS both caused a concentration-dependent increase in cytotoxicity after 24 hr as determined by MTT assay (Figure 6). Parallel cultures of cells in log-phase growth (i.e., cells exposed in medium containing 10% fetal bovine serum) were pretreated with either 50 µM PD98059 (MEK/ERK inhibitor) or dimethylsulfoxide vehicle control for 1 hr before treatment with H2O2 or LPS. Pretreatment of XTC-2 cells with PD98059 significantly reduced the cytotoxic response to either H2O2 or LPS (Figure 6). Marginal cytotoxicity was observed in quiescent cultures of XTC-2 cells treated with H2O2 or LPS, indicating that co-stimulation of cells with growth-stimulatory factors in serum plus the environmental stressor (H2O2 or LPS) were required for a maximal cytotoxic response. These data indicated that the cytotoxic effects of reactive oxygen species or bacterial endotoxin are mediated at least in part through activation of the ERK pathway. Significant differences between control versus treatment with PD98059 were determined by paired t-tests.

**Discussion**

In this study, we found that the ERK-2 MAP kinase pathway in Xenopus (XTC-2) cells is activated by oxidative stress (H2O2) or bacterial lipopolysaccharide (LPS). Moreover, ERK activation mediated, at least in part, the cytotoxic response to either H2O2 or LPS. This in vitro model of MAP kinase activation could help identify pollutants in aquatic ecosystems that contribute to the problem of amphibian malformations and population decline. Amphibians have evolved strategies to cope with harsh changes in natural conditions, including desiccation of temporary water sources (24). Despite this resilience in the face of natural environmental changes, amphibians may be particularly susceptible to a wide range of environmental toxicants that are present in polluted waters. The XTC-2 cell culture model might be useful for detecting cellular stress induced by pollutants that generate reactive oxygen species (e.g., metals, pesticides) or gram-negative bacteria that release endotoxin.

The XTC-2 in vitro model for measuring ERK-2 activation could also be used to test the effects of UV radiation generated in the laboratory. UV radiation has been proposed as a major stress factor for some amphibian populations (4, 16, 19). Moreover, it is important to consider that UV radiation...
may alter the chemical structure of some xenobiotics and thereby enhance toxicity. For example, Zaga and colleagues (25) reported that UV radiation enhanced the toxicity of carbaryl (a broad spectrum insecticide) to *Xenopus laevis* and *Hyla versicolor* embryos and tadpoles. Therefore, future investigation should include studies on agents such as carbaryl that are photoactivated by UV radiation to determine if this enhances the cellular stress response (e.g., ERK-2 activation) in cultured *Xenopus* cells.

The activation of MAP kinase signaling pathways in response to endogenous growth factors is well established and produces cell growth or differentiation (26,27). A variety of polypeptide growth factors (e.g., fibroblast growth factor-2 (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF)) bind to specific cell-surface receptors and initiate an intracellular signal transduction cascade involving the sequential phosphorylation of Ras, Raf, Map kinase kinase (MEK), and ERK. ERK then translocates to the nucleus, where it phosphorylates a variety of transcription factors that control gene expression (9). *Xenopus* oocyte maturation requires phosphorylation and activation of p42 MAP kinase, and the corresponding dephosphorylation and inactivation of the enzyme is crucial for the transition of the zygote from meiosis through the first phases of mitosis (10). Likewise, Bitangcol et al. (28) found that p42 MAP kinase activation regulates the normal progression through the cell cycle and is an essential component of the spindle assembly checkpoint. Furthermore, during *Xenopus* development, ordinary mesoderm differentiation relies on MAP kinase activation in response to FGF. However, overexpression of *Xenopus* MAP kinase phosphatase obstructs mesoderm induction by FGF and produces characteristic defects in mesoderm formation in intact embryos (29).

We postulate that oxidative or bacterial stress that activates the ERK-2 pathway during development could contribute to the morphologic anomalies seen in frogs, especially those involving mesodermal derivatives such as bone and muscle differentiation in the limbs. In fact, our cytotoxicity data showed that H2O2 or LPS caused more toxicity when cells were exposed in log-phase growth (i.e., in the presence of medium containing 10% fetal bovine serum) compared to cells going mitosis appear to be more susceptible to environmental stress. For example, H2O2 causes a cytotoxic response of mammalian connective tissue cells via the EGF receptor pathway leading to ERK activation, and a metabolic inhibitor of the ERK pathway (PD98059) blocked this cytotoxic response (23,30). We also observed that pretreatment of XTC-2 cells with PD98059 reduced the cytotoxic response of these cells to either H2O2 or LPS (Figure 6). Therefore, these environmental agents appear to cause XTC-2 cell death in part via activation of ERK-2.

Our data show that both oxidative and bacterial stress trigger ERK phosphorylation in a time- and concentration-dependent manner. H2O2 caused a rapid and more pronounced phosphorylation of ERK-2, because maximal phosphorylation took place at 30 min (Figure 2) with a 20-fold increase from the control (Figure 3). In contrast, LPS was a weaker activator of ERK-2. The maximum phosphorylation response occurred after 60 min (Figure 4) with only a 5- to 10-fold increase from the control (Figure 5). These differences are likely due to different mechanisms of ERK activation by H2O2 and LPS. As mentioned above, H2O2 crosses the cell membrane and activates the intracellular domain of the EGF receptor, resulting in downstream activation of Ras, Raf, MEK, and ERK (30). In contrast, LPS docks to a cell-surface receptor, mCD14, which then complexes with a transmembrane signaling receptor, the toll-like receptor 4 (TLR4) (31). Activation of TLR4 then leads to ERK activation through a mechanism that is not yet clear (32). The different time-course response of ERK-2 to H2O2 in comparison to LPS could allow for discrimination of...
oxidative and bacterial stress in samples obtained from polluted waters.

Other types of pollution in aquatic systems should be considered as stressors for MAP kinases. For example, metals may also represent a source of oxidative stress in polluted, aquatic environments (33). We have observed that vanadium pentoxide, a transition metal released from the industrial burning of fuel oil, causes ERK-2 activation in XTC-2 cells (data not shown). Also, pesticides have been associated with amphibian population declines (33). Certain pesticides recently have been reported to activate MAP kinase signaling pathways in a mammalian prostate cancer cell line (34). Therefore, it would be important to learn whether ERK-2 is activated by pesticides in the XTC-2 cell culture model.

In summary, we have shown that representational oxidative and bacterial agents cause activation of the ERK-2 MAP kinase in cultured *Xenopus* XTC-2 cells. H2O2 and LPS caused different temporal and dose–response patterns of ERK-2 activation. Blocking ERK-2 activation with the MEK inhibitor PD98059 partially protected XTC-2 cells from the cytotoxic effects of H2O2 or LPS. We propose that ERK-2 activation is a valuable biochemical marker for assessing factors that cause cellular stress in amphibians. Moreover, the XTC-2 cell culture model could be useful for rapidly assessing the “amphibian stress potential” of polluted waters. Future studies will address the utility of MAP kinase activation in XTC-2 cells as a screening assay for stressors present in the environment.

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