Poly(ADP-ribose) Polymerase-1 Signaling to Mitochondria in Necrotic Cell Death Requires RIP1/TRAF2-mediated JNK1 Activation*

Yue Xu†, Shuang Huang‡, Zheng-Gang Liu§, and Jiahuai Han†

From the †Department of Immunology, The Scripps Research Institute, La Jolla, California 92037 and ‡Cell and Cancer Biology Branch, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892

Poly(ADP-ribose) polymerase-1 (PARP-1) hyperactivation-induced necrosis has been implicated in several pathophysiological conditions. Although mitochondrial dysfunction and apoptosis-inducing factor translocation from the mitochondria to the nucleus have been suggested to play very important roles in PARP-1-mediated cell death, the signaling events downstream of PARP-1 activation in initiating mitochondria dysfunction are not clear. Here we have been suggested to play very important roles in PARP-1-mediated necrosis, in which the cell actively destroys itself while maintaining plasma membrane integrity, thus permitting non-inflammatory phagocytosis of the dying cell. Necrosis, on the other hand, has traditionally been regarded as a passive and unregulated form of cell death with morphology of cell swelling, loss of plasma membrane integrity, and the release of cellular contents into the extracellular environment, thus triggering an inflammatory response (11). PARP-1-mediated cell death is caspase-independent and lacks many morphological features of classic apoptosis considered as necrosis (4, 12). However, unlike “accidental” necrosis, it has recently been reported to be a regulated necrosis, in which the cell actively initiates its death program after PARP-1 hyperactivation (13).

Despite its pathophysiological importance, the cell death signaling pathway induced by PARP-1 activation is still not clear. It has been reported that PARP-1 hyperactivation prompts mitochondria dysfunction, which in turn releases apoptosis-inducing factor (AIF) from the mitochondria to the nucleus (14, 15). Blockage of these events dramatically prevents cell death induced by PARP-1 activation, suggesting that mitochondria change is a key event of cell death downstream of PARP-1 hyperactivation. However, the mechanism by which PARP-1 activation leads to mitochondrial dysfunction and release of AIF is largely unknown. c-Jun N-terminal kinases (JNK) are a group of mitogen-activated protein kinases (MAPKs) that have recently been demonstrated to be involved in necrosis by several groups of investigators (16–18). Receptor-interacting protein 1 (RIP1) and tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) are known to play important roles in cellular responses to TNF and TNF family members, and both were shown to be required for TNF-induced necrotic cell death (19–22). However, whether JNK, RIP1, and TRAF2 are involved in PARP-1-mediated necrotic cell death has not been studied.

N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) is known to activate PARP-1, and the activated PARP-1 causes cell death in a variety of different cell types. To investigate the signaling pathway downstream of PARP-1 in mediating mitochondrial dysfunction, we examined the involvement of different MAPKs, as well as RIP1 and TRAF2, in MNNG-treated MEF cells. We found that JNK, RIP1, and TRAF2 all participated in PARP-1-mediated mitochondria dysfunction and subsequent cell death. Our data showed that MNNG-induced cell death requires the following signal pathway: PARP-1 > RIP1/TRAF2 > JNK1 > mitochondrial dysfunction.

* This work was supported in part by National Institutes of Health Grants AI41637, AI054796, GM67101, and GM37696. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Immunology, The Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-8719; Fax: 858-784-8665; E-mail: mcbxuyue@scripps.edu.

‡ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; AIF, apoptosis-inducing factor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; CSA, cyclosporin A; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)-butoxy]-1(2H)-isoquinolinone; MEF, mouse embryonic fibroblast; WT, wild type; PI, propidium iodide; PBS, phosphate-buffered saline; TMRM, tetramethylrhodamine methyl ester.

8788 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 13 • MARCH 31, 2006

Printed in the U.S.A.
**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**—Anti-phospho-JNK1/2, phospho-extracellular signal-regulated kinase, and phospho-p38 were obtained from Cell Signaling. Anti-FLAG (M2) and actin antibodies were bought from Sigma. Anti-PAR, RIP1, and TRAF2 were purchased from Pharmingen. AIF antibody was from Santa Cruz Biotechnology. The mammalian expression plasmids for TRAF2 and RIP were gifts from Dr. Zheng-gang Liu. P38 inhibitor SB203580, JNK inhibitor SP600125, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 were purchased from Calbiochem. MNNG, cyclosporin A (CsA), 3,4-dihydro-5-[4-(1-piperidinyl)-butoxy]-1(2H)-isooquinolinone (DPQ), and 1,5-dihydroxyisoquinoline were obtained from Sigma.

**Cell Culture and Transfection**—RIP1−/−, TRAF2−/−, and TNFR1−/− mouse embryonic fibroblast (MEF) cells, as well as each wild-type (WT) cell line from the same background (C57BL/6J) as the knock-out cells, were described elsewhere (22). JNK1−/−, JNK2−/−, and WT cells from the same background (C57BL/6j) as the knockouts were obtained from Dr. M. Karin. These cells had been immortalized when we received them and passed for 3–4 generations in our laboratory. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells with the same passage number were used for each experiment. For MNNG treatment, cells were treated with MNNG for 10 min. Cells were washed and fed with fresh medium with no MNNG and cultured for the indicated periods of time. For transfection, cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Reconstituted cell lines were selected with hygromycin.

**Cell Viability Assay**—Plasma membrane integrity was assessed by determining the ability of the cells to exclude propidium iodide (PI; Sigma). Cells were trypsinized, collected by centrifugation, washed once with PBS, and resuspended in PBS containing 1 μg of PI/ml. The level of PI incorporation was quantified by flow cytometry on a FACScan flow cytometer. PI-negative cells with normal size were considered to be live cells.

**Mitochondrial Membrane Potential**—Cells were treated with different combinations of chemicals and 5 μM 5,5′,6′,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1; Molecular Probes) for 1 h. Nuclei were stained with PI (Sigma) for 2 min after secondary antibody conjugated with fluorescein isothiocyanate (Molecular Probes) were washed with blocking solution three times and incubated with the secondary antibody conjugated with fluorescein isothiocyanate (Molecular Probes) for 1 h. Nuclei were stained with PI (Sigma) for 2 min after secondary antibody incubation and two rinses with PBS. After washing three times with PBS, coverslips were mounted onto microscope slides and the slides were analyzed using a fluorescence microscope.

**Immunocytochemistry**—Cells after treatment were washed twice with ice-cold PBS before fixation with ice-cold methanol. After blocking with 2% bovine serum albumin in PBS containing 0.2% Triton X-100, cells were incubated with the primary antibody against AIF for 1 h. Cells were washed with blocking solution three times and incubated with the secondary antibody conjugated with fluorescein isothiocyanate (Molecular Probes) for 1 h. Nuclei were stained with PI (Sigma) for 2 min after secondary antibody incubation and two rinses with PBS. After washing three times with PBS, coverslips were mounted onto microscope slides and the slides were analyzed using a fluorescence microscope.

**Western Blot Analysis**—After treatment with different reagents as described in the figure legends, cells were collected and lysed in lysis buffer (20 mM Tris, pH 7, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate). 50 μg of the cell lysate from each sample were fractionated by SDS-PAGE and Western blotted. The proteins were visualized by enhanced chemiluminescence (ECL), according to the manufacturer’s (Amersham Biosciences) instruction.

**RESULTS**

**JNK Is Required for PARP-1-mediated Cell Death**—We treated MEF cells with MNNG, a DNA alkylating agent widely used to activate PARP-1 in studying PARP-1-mediated cell death (4, 14). As shown in Fig. 1a, MNNG treatment induces massive cell death within 24 h and pretreatment of cells with the PARP-1 inhibitors DPQ and 1,5-dihydroxyisoquinoline (DHIQ) significantly blocks MNNG-induced cell death, thus confirming that PARP-1 activation is mainly responsible for the cytotoxicity of MNNG. We chose MEF cells to study PARP-1-mediated cell death because this enabled us to utilize gene knock-out cells. First, we tested whether PARP-1 hyperactivation led to non-apoptotic cell death in MEF cells as in other cells (3, 4, 12). We treated MEF cells with MNNG and found that no caspase 3 or caspase 8 activation was detected following MNNG treatment (data not shown). Caspase inhibitors (benzylxoycarbonyl-VAL and benzlyoxycarbonyl-DEVD) were also unable to block PARP-1-mediated cell death (Fig. 1b). We also did not observe classical apoptosis features such as membrane blebbing and chromatin condensation after MNNG treatment (data not shown). Collectively, our data confirm that PARP-1-induced cell death in MEF cell is non-apoptotic.

MAPKs play a very important role in the stress response (23). As shown in Fig. 1c, stimulation of cells with MNNG resulted in a very rapid and persistent activation of the p38 and JNK pathways as determined by the detection of activating phosphorylation of these kinases. To test whether MAPKs play any role in PARP-1-mediated caspase-independent cell death, we pretreated wild type mouse fibroblast cells with a specific set of MAPK inhibitors and followed with MNNG treatment. As shown in Fig. 1d, the pretreatment of cells with compounds SB203580 and U0126, inhibitors of p38 and extracellular signal-regulated kinase, respectively, did not alter the sensitivity of cells to MNNG-mediated cell death, thus confirming that PARP-1 activation is mainly responsible for MNNG-induced caspase-independent cell death when compared with control MeSO pretreatment. By contrast, pretreatment with SP600125, an inhibitor of JNK, caused significant resistance to MNNG-induced cell death. To confirm the requirement of JNK in MNNG-induced cell death, we compared MNNG-induced cell death in WT and either p38−/−, JNK1−/−, or JNK2−/− MEF cells. JNK1−/− cells were found to be resistant to MNNG killing when compared with WT MEF cells. JNK2−/− cells were also resistant to MNNG cytotoxicity but to a lesser extent. (Fig. 1e). By contrast, p38−/− cells were even a little more sensitive to MNNG-induced cell death than WT cells. Therefore, JNK activation plays an important role in MNNG-induced caspase-independent cell death.
PARP-1-induced Cell Death Requires JNK Activation

FIGURE 1. JNK is required for PARP-1-mediated necrosis. a, WT MEF cells were pretreated with DPQ (50 μM) or 1,5-dihydroxyisoquinoline (DHIQ) (150 μM) and exposed to MNNG (0.5 mM) or remained untreated. 24 h after MNNG exposure, cell death was measured by PI exclusion. Data shown are the average of the results of three independent experiments. b, WT MEF cells were pretreated with benzoyloxycarbony-VAD (50 μM) or benzoyloxycarbony-DEVD (100 μM) and exposed to MNNG (0.5 mM) or remained untreated. After 24 h, cell death was measured as in panel a. c, WT MEF cells were treated with MNNG (0.5 mM) for the indicated time periods. Cell extracts were Western blotted for phospho-p38, JNK, and actin. d, MEF cells were treated with MNNG or remained untreated for 15 min. Cell extracts were Western blotted by anti-poly(ADP-ribose) (PAR) antibodies.

JNK Is Downstream of PARP-1 Activation—The requirement of JNK for MNNG-induced cell death indicates that JNK may either be involved in PARP-1 activation upon MNNG stimulation or it may function downstream of PARP-1 activation. To distinguish between these two possibilities, we first compared PARP-1 activation by detecting poly(ADP-ribose) formation in WT MEF and JNK1−/− MEF cells. We found that there was no difference between these two cell lines (Fig. 2a), excluding the possibility that JNK1 is required for PARP-1 activation. Moreover, JNK activation by MNNG treatment was impaired when specific PARP-1 inhibitors were applied, suggesting that JNK activation is downstream of PARP-1 activation (Fig. 2b).

Mitochondrial Membrane Depolarization Is Required for PARP-1-mediated MEF Cell Death—It has been reported previously that PARP-1 hyperactivation leads to mitochondrial membrane depolarization and that this depolarization is required for PARP-1-mediated cell death (15). To determine whether PARP-1 activation induces Δψm depolarization in MEF cells, we stained cells with TMRM, a cell-permeable fluorescent dye that accumulates in mitochondria proportionally with Δψm (24, 25). As shown in Fig. 3a, MNNG treatment led to a decrease in TMRM fluorescence indicative of Δψm depolarization and this Δψm depolarization was PARP-1-mediated, because pretreatment of cells with the PARP-1 inhibitor DPQ significantly attenuated depolarization. Next, we tested whether Δψm depolarization is required for PARP-1-induced cell death. We first treated cells with the potent mitochondrial permeability transition inhibitor CsA (26), followed by treatment with MNNG. CsA dramatically inhibited the mitochondrial membrane depolarization induced by MNNG, and it also efficiently suppressed MNNG-induced cell death (Fig. 3a and b). CsA did not inhibit PAR formation (Fig. 3c), thereby excluding the possibility that CsA blocks PARP-1-mediated death by inhibiting PARP-1 activity. Together, these results indicate that mitochondrial membrane depolarization is an essential step in PARP-1-mediated cell death in MEF cells. Because our experiments involved long incubation times, we could not include TMRM during the entire duration of our experiments. As a result, the mitochondrial membrane potential measured may not be absolutely accurate.

Δψm Depolarization by PARP-1 Activation Requires JNK Activity—We then examined whether MNNG-induced Δψm depolarization requires the presence of JNK by using the JNK inhibitor, SP600125. Δψm was monitored using the fluorescent dye JC-1 (27). JC-1 stains mitochondria differentially
in accordance with their Δψm. Active mitochondria with high Δψm accumulate red JC-1 aggregates, whereas mitochondria with low Δψm (inactive) display the green monomeric form of JC-1. As shown in Fig. 4a, MNNG treatment induced a marked reduction in the ratio of red to green fluorescence, indicating a fall in Δψm. Interestingly, this mitochondrial membrane depolarization was efficiently blocked by Sp600125. To further confirm the important role of JNK in MNNG-induced depolarization, we compared MNNG-induced Δψm changes in WT and JNK1−/− MEF cells. As shown in Fig. 4b, MNNG treatment led to a significant decrease in the ratio of red to green fluorescence in WT MEF cells, whereas this decrease was reduced in JNK1−/− MEF cells. As a control, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, uncoupler of mitochondrial oxidative phosphorylation, was applied to MEF cells. Both JNK1−/− and WT MEF cells showed comparable mitochondrial depolarization, suggesting that JNK1−/− cells are not inherently resistant to Δψm depolarization. Fig. 4c shows representative images captured 12 h after MNNG treatment. After MNNG treatment, mitochondria showed an increase in monomer green fluorescence and a decrease in JC-1 aggregated red fluorescence, indicating Δψm depolarization. This change was not observed in JNK1−/− MEF cells or the WT cells treated with the PARP-1 inhibitor DPQ. Together, these results demonstrate that JNK is crucial to PARP-1-induced mitochondrial membrane depolarization.

**JNK Is Required for AIF Translocation**—The mitochondria-localized flavoprotein AIF is known as a mediator for PARP-1-induced cell death (14, 28). In response to PARP-1 hyperactivation, AIF is released from the mitochondria and subsequently translocates into the nucleus. Blockage of AIF translocation has been shown to inhibit PARP-1-mediated cell death, suggesting that AIF translocation is an essential step in the cell death process (14). Therefore, we investigated whether JNK was also required for AIF translocation. Co-labeling of AIF with PI, which stains the nucleus, after 12 h of MNNG treatment showed that AIF translocated to the nucleus after PARP-1 activation and this translocation was blocked by the PARP-1 inhibitor (Fig. 5a). AIF translocation was also prevented in cells treated with CsA, indicating that AIF translocation requires Δψm depolarization. More importantly, AIF translocation was not observed in JNK1−/− MEF cells, suggesting that JNK activation is required for AIF translocation after MNNG exposure. This view was further confirmed by subcellular fractionation studies. As shown in Fig. 5b, AIF was redistributed from the mitochondria to the nucleus after MNNG treatment of MEF cells but not in cells pretreated with the JNK inhibitor.

**MNNG-induced Cell Death Requires RIP1 and TRAF2**—To better understand how PARP-1 activation induces cell death, we thought that it necessary to know what other signaling regulators are involved in PARP-1-mediated JNK activation. RIP1 and TRAF2 are known downstream effectors of the TNF receptor and are required for TNF-induced JNK activation (19, 20). RIP1 and TRAF2 are also known to play an essential role in some cases of necrotic cell death (21, 22, 29, 30). We felt that RIP1 and TRAF2 may be associated with PARP-1-induced JNK activation and/or cell death. To examine whether RIP1 and TRAF2 participate in PARP-1-mediated cell death, we compared MNNG-induced cell death in WT and RIP1−/− or TRAF2−/− cells. RIP1−/− and TRAF2−/− cells are resistant to MNNG-induced cell death in comparison with WT MEF cells, suggesting that RIP1 and TRAF2 are required for MNNG-induced cell death (Fig. 6a). Because PARP-1 activation by MNNG is equal in knock-out and WT cells, the resistance of RIP1−/− and TRAF2−/− cells to MNNG killing cannot be because of impaired PARP-1 activation (Fig. 6b). Because RIP1 and TRAF2 are downstream effectors of the TNF receptor, it is possible that MNNG induces TNF through RIP1 and TRAF2 to trigger cell death. To investigate this possibility, we examined the sensitivity of TNFR1−/− MEF cells to MNNG treatment and found that it is comparable with that of WT MEF cells (Fig. 6c). As expected, TNFR1−/− MEF cells are resistant to TNF-induced cell death (Fig. 6c). These results exclude the possibility that RIP1 and TRAF2 function in the TNF signaling pathway in MNNG-treated cells. To unambiguously determine that RIP1 and TRAF2 are required for MNNG-induced cell death, we generated stable cell lines from RIP1−/− and TRAF2−/− cells by ectopically expressing RIP1 and TRAF2 in their corresponding knock-out cells (Fig. 6d) and examined the susceptibility of each reconstituted cell line to MNNG-induced cell death (Fig. 6e). The reconstituted RIP1−/− and TRAF2−/− cells showed restored sensitivity to MNNG-induced cell death (Fig. 6e), confirming that RIP1 and TRAF2 are indeed required for MNNG-induced cell death. Because RIP1 and TRAF2 have no effect on PARP-1 activation and they are required for MNNG-induced cell death, we concluded that RIP1 and TRAF2 are either downstream of, or parallel with, PARP-1 activation in MNNG-treated MEF cells.

**Δψm Depolarization and AIF Translocation by PARP-1 Activation Require RIP1 and TRAF2**—As shown above, Δψm depolarization is a key event leading to cell death induced by PARP-1 activation. Next, we sought to examine whether RIP1 and TRAF2 participate in Δψm changes. As expected, activation of PARP-1 led to a decrease in the ratio of JC-1 590/535 in WT cells, indicating depolarization. By contrast, this Δψm depolarization was inhibited in RIP1−/− and TRAF2−/− cells (Fig. 7a). Fig. 7b shows representative JC-1 images captured after MNNG treatment. In WT cells, a dramatic decrease of J-aggregate fluorescence was observed after MNNG induction. However, J-aggregate fluorescence was not affected by MNNG stimulation in RIP1−/− and

![Image](image-url)
TRAF2−/− cells, suggesting that mitochondria remained intact in these two knock-out cells. AIF translocation following PARP-1 activation was also investigated using a fluorescence microscope. As shown in Fig. 7c, after MNNG treatment AIF was translocated from the mitochondria to the nucleus in WT cells, but in RIP1−/− and TRAF2−/− cells translocation was blocked. This was further confirmed by subcellular fractionation studies (Fig. 7d). Altogether, these results suggest that RIP1 and TRAF2 are required for ΔΨm depolarization and AIF translocation induced by PARP-1 activation.

**DISCUSSION**

Hyperactivation of PARP-1-mediated caspase-independent cell death has been implicated to play a role in some pathophysiological processes such as ischemia and inflammation. Although mitochondrial membrane depolarization and the subsequent translocation of AIF from the mitochondria to the nucleus are thought to be necessary for PARP-1-elicited death (14, 15), the signaling events between PARP-1 and JNK1 activation upon PARP Activation are not well understood.
activation and mitochondria dysfunction are not clear. We have shown in this report that JNK, but not p38 or extracellular signal-regulated kinase, is essential for PARP-1-induced cell death. In addition, we found that RIP1 and TRAF2 are also required for PARP-1-mediated cell death. Our study revealed that RIP1 and TRAF2 are downstream of PARP-1 in mediating JNK activation and that JNK activation is required for mitochondrial depolarization, AIF translocation, and subsequent cell death in PARP-1-hyperactivated MEF cells. Thus, we for the first time elucidated some of the signaling events between PARP-1 overactivation and mitochondria dysfunction.

It has been well established that the JNK and p38 pathways are crucial for cellular responses to stress (23). The requirement of these kinases for cell death has been observed in cells treated with stress stimuli (33–35). Recently, the role of JNK in cell death has been highlighted by its opposite contribution to apoptosis and necrosis induced by TNF (16). In this study, we have shown that PARP-1-mediated cell death specifically requires JNK activation but not p38 or extracellular signal-regulated kinase. This conclusion was based on the observation that the pharmacological inhibition of JNK by the specific chemical inhibitor SP600125 and the genetic ablation of JNK1 significantly enhance the cell survival rate upon PARP-1 activation. In support of our result that JNK1 plays an important role in PARP-1-mediated cell death, several studies showed that JNK is involved in ischemia/reperfusion-induced neuronal death, in which PARP-1 activation has been strongly implicated (36, 37). JNK mediates cell death via transcription-dependent or -independent pathways (38–40). Addition of cycloheximide showed no effect on MNNG-induced cell death, suggesting that the transcription-regulating role of JNK is not important in PARP-1-mediated cell death (data not shown). By contrast, we found that JNK regulates the mitochondrial membrane potential change and the translocation of AIF from the mitochondria to the nucleus after PARP-1 induction. Indeed, JNK has been shown to regulate the mitochondrial function in several settings (34, 41–44). Bcl-2 family members are known as major mediators in regulating mitochondrial membrane permeability in response to different death stimuli. Bcl-2 and Bcl-xL can be phosphorylated by JNK, and this phosphorylation suppresses their anti-apoptotic functions (42–44). A caspase-independent processing of Bid, a pro-apoptotic member of the Bcl-2 family, in response to TNF has also been suggested to require JNK activation (45). Therefore, it is likely that, in response to PARP-1 activation, JNK regulates mitochondrial function through modifications of Bcl-2 family members. Future studies along this line would clarify the molecular mechanism by which JNK regulates the mitochondrial potential in PARP-1-hyperactivated cells.

RIP1 and TRAF2 were initially identified as important downstream molecules of TNFR1 signaling. The recruitments of RIP1 and TRAF2 mediate NF-κB and MAPK activation. Neither RIP1 nor TRAF2 is essential for TNF-induced apoptosis; instead, they act mainly as survival signals against TNF-induced apoptosis (19, 46). However, recent studies have suggested that RIP1 and TRAF2 are essential for necrosis induced by various stimuli such as TNF, H2O2, and arsenic, indicating a common role of RIP1 and TRAF2 in the regulation of necrosis (21, 22, 29, 30). Consistent with this view, data from our study suggest that caspase-independent cell death induced by PARP-1 also requires the function of RIP1 and TRAF2. RIP1 and TRAF2 regulate PARP-1-induced-necrosis by influencing JNK activity, which appears to be a similar case in TNF- and H2O2-induced necrosis. TNF-
H$_2$O$_2$-, and arsenic-induced necrosis have in common a relationship with reactive oxygen species. Both TNF and arsenic stimulation can generate reactive oxygen species, which is essential for their death (22, 47). It is known that reactive oxygen species is one of the major causes of DNA damage, which in turn activates PARP-1. Moreover, the involvement of PARP-1 activation in TNF-, H$_2$O$_2$-, and arsenic-induced necrosis has also been suggested (14, 47, 48). Thus, TNF-, H$_2$O$_2$-, and arsenic-induced necrosis may share a common death pathway, the PARP-1-mediated death pathway, in which JNK, RIP1, and TRAF2 are required. However, one of the most important questions yet to be

**FIGURE 7.** RIP1 and TRAF2 mediate Δψm decrease and AIF translocation. a, WT MEF, RIP1−/−, or TRAF2−/− cells were treated with MNNG for different periods of time, and Δψm was measured with a flow cytometer by using JC-1 dye. b, WT MEF, RIP1−/−, or TRAF2−/− cells were treated with MNNG; 12 h after MNNG exposure, cells were stained with JC-1. Representative JC1 images are shown. Scale bar, 5 μm. c, WT MEF, RIP1−/−, or TRAF2−/− cells were treated with MNNG as above, and AIF translocation was examined by fluorescence microscope. Scale bar, 5 μm. d, cells were treated as in panel c, and nuclear fractions were Western blotted with AIF antibodies. Ref1 was used as a nuclear marker.

**FIGURE 8.** Impaired JNK activation in RIP1−/− or TRAF2−/− cells. a, WT, RIP1−/−, or TRAF2−/− MEF cells were treated with MNNG for the indicated time periods. Cell extracts were Western blotted for phospho-JNK. b, our proposed signaling pathway for PARP-1-mediated cell death is shown. PARP-1-mediated cell death was achieved by RIP1, TRAF2, and JNK-regulated mitochondrial membrane depolarization.
addressed is how intracellular molecules like RIP1 and TRAF2 sense PARP-1 activation, because the TNF receptor is not involved. Hyperactivation of PARP-1 is known to deplete intracellular energy. RIP1 and TRAF2 may sense this energy change or there may exist a major energy sensor molecule that may recruit RIP1 and TRAF2 to activate JNK. Possible candidates include cAMP-dependent kinase or Tor signaling, and future research would benefit greatly by investigating these pathways.

Prior studies have documented the important role of mitochondria in PARP-1-induced cell death. However, only recently has it been suggested that NAD$^+$ is an essential link between PARP-1 activation and mitochondrial potential change (15). External delivery of NAD$^+$ could prevent PARP1-induced mitochondrial changes and cell death, highlighting the role of NAD$^+$ in regulating the mitochondria. How NAD$^+$ regulates the mitochondria remains an unsolved question. Several indirect mechanisms have been proposed. First, NAD$^+$ synthesis consumes ATP and it is known that ATP and ADP are important inhibitors for mitochondria permeability transition (49). Moreover, NAD$^+$ is essential for glycosis; thus, depletion of NAD$^+$ may impair glycolytic flux to the mitochondria and this impaired substrate transport may also lead to mitochondria depolarization (50). Finally, our study has shown that JNK is involved in the regulation of mitochondria change, providing another mechanism by which NAD$^+$ may regulate the mitochondria through the RIP, TRAF2, and JNK pathway. It is likely to be true, but how NAD$^+$ regulates the RIP1, TRAF2, and JNK pathway must be further addressed.

In conclusion, our results reveal a signaling pathway induced by PARP-1 in which RIP1, TRAF2, and JNK regulate mitochondrial function and cell death induced by PARP-1 hyperactivation (Fig. 8b). Specific inhibitors targeted on this pathway could provide future clinical benefits to patients in a variety of pathophysiologic conditions.

Acknowledgments—We thank Dr. M. Karin for kindly providing JNK1−/− and JNK2−/− cells and Dr. A. Nebreda for p38−/− cells.

REFERENCES

1. D’Amours, D., Desnoyers, S., D’Silva, I., and Poirier, G. G. (1999) Biochem. J. 342, Pt. 2, 249–268
2. de Mucia, G., Schreiber, V., Molinete, M., Saulier, B., Poch, O., Masson, M., Nieder- gang, C., and Menissier de Murcia, J. (1994) Mol. Cell. Biochem. 138, 15–24
3. Berger, N. A. (1985) Radiat. Res. 101, 4–15
4. Ha, H. C., and Snyder, S. H. (1999) J. Biol. Chem. 274, 1634–1635
5. Virag, L., and Szabo, C. (2002) J. Biol. Chem. 277, 11671–11673
6. Zong, W. X., Ditsworth, D., Bauer, D. E., Wang, Z. Q., and Thompson, C. B. (2004) Science 263, 687–689