Manganese(II) Induces Apoptotic Cell Death in NIH3T3 Cells via a Caspase-12-dependent Pathway*

Received for publication, April 9, 2002, and in revised form, April 15, 2002 Published, JBC Papers in Press, April 18, 2002, DOI 10.1074/jbc.C200226200

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Under physiological conditions, manganese(II) exhibits its catalase-like activity. However, at elevated concentrations, it induces apoptosis via a non-mitochondria-mediated mechanism (Oubrahim, H., Stadtman, E. R., and Chock, P. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9505–9510). In this study, we show that the Mn(II)-induced apoptosis, as monitored by caspase-3-like activity, in NIH3T3 cells was inhibited by calpain inhibitors I and II or the p38 MAP kinase inhibitor, SB202190. The control experiments showed that each of these inhibitors in the concentration ranges used exerted no effect on activated caspase-3-like activity. Furthermore, caspase-12 was cleaved in Mn(II)-treated cells, suggesting that the Mn(II)-induced apoptosis is mediated by caspase-12. This notion is confirmed by the observations that pretreatment of NIH3T3 cells with either caspase-12 antisense RNA or dsRNA corresponding to the full-length caspase-12 led to a dramatic decrease in caspase-3-like activity induced by Mn(II). The precise mechanism by which Mn(II) induced the apoptosis is not clear. Nevertheless, Mn(II), in part, exerts its effect via its ability to replace Ca(II) in the activation of m-calpain, which in turn activates caspase-12 and degrades Bcl-xL. In addition, the dsRNA method serves as an effective technique for knocking out caspase-12 in NIH3T3 cells without causing apoptosis.

Apoptosis is the physiological form of programmed cell death that serves to remove damaged and unwanted cells and to maintain tissue homeostasis. It is characterized by a series of distinct morphological and biochemical changes (1, 2). Proteases, particularly the family of cysteine proteases called caspases, have been shown to play critical roles in cellular execution of apoptosis (3–6). To date, 14 caspases have been identified. Most are located in the cytosol as zymogens, where they are activated by apoptotic stimuli-mediated signaling cascades (3, 4, 6). Caspase-8 mediates apoptotic signals from death receptors on plasma membranes (7, 8), caspase-9 plays a key role in mitochondria-mediated apoptosis (9), and caspase-12, which can be activated in cells by β amyloid peptide and respiratory syncytial virus, mediates endoplasmic reticulum (ER)1-specific apoptosis (10). ER regulates a number of cellular functions, including cellular responses to stress and intracellular Ca(II) levels. Elevation of intracellular Ca(II) and oxidants have been shown to cause ER stress and ultimately lead to apoptosis (11, 12).

Under physiological conditions, Mn(II) has been shown to exhibit catalase-like activity (13, 14) and is capable of protecting endothelial cells from H₂O₂ toxicity and from reactive oxygen species (ROS) generated during oxidative burst of neutrophils (15, 16). We have shown that in the 0.5–2 mM range Mn(II) induced mitochondria-independent apoptosis in HeLa cells, and it also caused an elevation of ROS and Mn(II)-superoxide dismutase (17). Here we show by the dsRNA, and antisense RNA methods that the observed Mn(II)-induced apoptosis is mediated by caspase-12 known to localize in the ER.

EXPERIMENTAL PROCEDURES

Materials—MnCl₂ 4H₂O (99.9%) (MnII) was from Aldrich. Materials for protein electrophoresis and poly(vindylidenedifluoride) membrane (0.2 μm) were purchased from Bio-Rad. The caspase-3 substrate, DEVD-AMC, was from PharMingen. Anti-caspase-12 antibody was kindly provided by J. Yuan of Harvard Medical School. Primers and antisense were synthesized by biosynthesis. Megascript T7 used for dsRNA synthesis was purchased from Ambion. Highly pure PCR product purification kits were from Roche Molecular Biochemicals, and penetratin 1 was from Ambigene. The inhibitors of p38 MAP kinase, SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)IH-imidazole) and calpain inhibitors I (N-Ac-Ile-norleucinal) and II (N-Ac-L-leucinal) were purchased from Calbiochem.

Cell Culture—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (4.5 g/liter, 25 mM) and supplemented with 10% (v/v) fetal bovine serum without heat inactivation, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. The medium was changed every other day. All Mn(II) treatments were performed in culture medium (DMEM) containing 10% serum. Just prior to treatment, the medium was removed and replaced with fresh medium.

RNA Isolation and RT-PCR—Total RNA was isolated from NIH3T3 cells using the TriZol reagent (Invitrogen) as described by the manufacturer. RT-PCR was performed using Superscript II (Invitrogen) on 5 μg of total RNA according to the manufacturer’s instructions. PCR reactions (100 μl) were performed with 2 μl of reverse transcriptase reaction mixtures that contained 0.5 μM of the forward 5'-ATGGCGG-GCCAGGAGGACATC-3' and reverse 5'-GGTGCTGTGCTAATTC-CCGG-3' primers to amplify caspase-12 to full length. PCR products were purified and resuspended in 10 μl of water.

dsRNA Production—Full-length caspase-12 cDNA was amplified by PCR. The primers used in the PCR reaction contained a 5' T7 RNA polymerase binding site (GAATTATACGACTCACTATAGGGA) followed by sequences specific for caspase-12 (forward, 5'-ATGGCGG-GCCAGGAGGACATC-3' and reverse, 5'-GGTGCTGTGCTAATTC-CCGG-3'). The PCR products were purified by using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). The purified PCR products were used as templates for the MegascriptT7 transcription kit (Ambion, Austin, TX) to produce dsRNA. The dsRNA products were precipitated with lithium chloride and resuspended in 1 mM Tris buffer. The dsRNAs were annealed by incubation at 65 °C for 60 min followed by slow cooling to room temperature for at least 50 min. The

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‡ The abbreviations used are: ER, endoplasmic reticulum; Mn(II), manganese; PVDF, polyvinylidene difluoride; AMC, 7-amino-4-methylcoumarin; dsRNA, double-stranded RNA; MAPK, mitogen-activated protein kinase; SB202190 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)IH-imidazole; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcriptase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline.
Calpain Inhibitors Protect NIH3T3 Cells against Mn(II)-induced Apoptosis—Activation of the p38 MAP kinase has been shown to precede apoptosis induced by stress in some cells and is associated with cytokine expression and proliferation in other cells (21). SB202190 is a specific inhibitor of p38 MAP kinase. To investigate whether Mn(II)-induced stress can be overcome by SB202190, we studied its effect on Mn(II)-mediated apoptosis in NIH3T3 cells. Fig. 2 shows that when NIH3T3 were incubated together with 0.5 mM Mn(II) and two different concentrations of SB202190 (1 and 2 μM) for 24 h, the caspase-3 activation was significantly inhibited relative to that observed with only Mn(II). However, the p38 inhibitor exerted no effect on caspase-3 activity in cells unless it was present during the Mn(II) treatment (Fig. 2). Moreover, it had no effect on caspase-3 activity induced by prior treatment with Mn(II) (data not shown).

Mn(II) Induces Caspase-12 Cleavage—Since the apoptosis induced by Mn(II) is not a mitochondria-mediated process (17), we investigated the possible involvement of caspase-12 in this apoptotic pathway. When NIH3T3 cells were treated with 0.5 or 1.0 mM Mn(II) for 24 h, we found that relative to the untreated cells, the 60-kDa procaspase-12 was reduced in a Mn(II) concentration-dependent manner (Fig. 3). This observation is consistent with the fact that Mn(II), in place of Ca(II), can activate m-calpain (data not shown). The reduction of procaspase-12 was also observed when the cells were incubated with 10 μM A23187 ionophore for 24 h (see Fig. 3), a condition known to induce apoptosis.

EVIDENCE FOR THE PARTICIPATION OF CASPASE-12—To study the involvement of caspase-12 in Mn(II)-mediated apoptosis in NIH3T3 cells, we interfered with the synthesis of caspase-12 by (i) transfecting the cells with caspase-12 antisense RNA and (ii) “knocking out” the enzyme using the dsRNA-mediated interference of gene expression method. When NIH3T3 cells were transfected with caspase-12 antisense RNA for 6 h and then treated with 0.5 mM Mn(II) for 24 h, a significant decrease in caspase-3 activity was observed in comparison to that found in the untransfected cells (Fig. 4, lanes 3 and 5). The caspase-3 activity of cells that were treated with caspase-12 antisense was comparable with that of non-treated cells. Moreover, Fig. 4, lane 4, shows that the scramble oligonucleotide was insuffi-
Caspase-3 activation. The control experiments showed that the cells exhibited almost total protection from Mn(II)-induced apoptosis, whereas the experimental caspase-12 antibody could not prevent Mn(II)-induced apoptosis in NIH3T3 cells. Cells were incubated with Mn(II) or A23187 Ca(II) ionophore for 24 h, and equal amounts of their lysates were loaded onto 4–20% gels. The proteins were transferred onto PVDF membrane, and caspase-12 was monitored using caspase-12 antibody.

Effect of caspase-12 antisense on Mn(II)-induced caspase-3 activation in NIH3T3 cells. Cells were incubated with either 5'-thiol-modified caspase-12 antisense (black bars) or with 5'-thiol-modified scramble oligonucleotide (gray bar) or no oligonucleotide (white bars). Six hours after transfection the cells were incubated for 24 h at 37 °C in either the absence (lanes 1 and 2) or presence (lanes 3–5) of 0.5 mM Mn(II). After incubation, caspase-3 activity was monitored as described in the legend to Fig. 1. FAU, fluorescence arbitrary unit.

DISCUSSION

Although Mn(II) in the micromolar concentration range can protect cells against oxidative stress, we previously showed that in high concentrations, e.g. 0.5–2 mM, Mn(II) induced HeLa cells to undergo apoptosis mediated by caspase-3 activation (17). At high Mn(II) concentrations, we also observed an elevation of ROS, as detected by intracellular 2',7'-dichlorodihydrofluorescein diacetate oxidation and Mn(II)-SOD generation. However, unlike most ROS-induced apoptosis, the apoptosis caused by elevated Mn(II) is not mediated by the mitochondrial pathway (17). Our current results indicate that the Mn(II)-induced activation of caspase-3 is mediated through caspase-12 activation, and caspase-12 is known to be associated with ER-mediated apoptosis (10–12). To investigate the role of caspase-12 in Mn(II)-induced apoptosis, we used NIH3T3 cells, because the apoptotic effect in HeLa cells was also observed in NIH3T3 cells, and the anti-caspase-12 antibody raised in mice works best with murine caspase-12.

Our results show that with antisense caspase-12 treatment, the Mn(II)-induced caspase-3 activation was reduced about 70% (Fig. 4), while knocking out caspase-12 with the dsRNAi technique essentially eliminated the Mn(II)-dependent caspase-3 activation (Fig. 5). Together, these data indicate that caspase-12 is required for the Mn(II)-induced apoptosis in NIH3T3 cells. It should be pointed out that the dsRNAi method is an effective technique for suppressing the expression of the DNA of a given protein. It provides a total knock-out of caspase-12 as shown in Fig. 5C and thus totally eliminated the Mn(II)-induced apoptosis (see Fig. 5D). The full-length dsRNA-treated cells maintained null caspase-12 conditions for at least 3 days without apoptosis.

Caspase-12 is localized on the cytosolic side of the ER membrane. It has been identified as an upstream caspase in the ER-mediated apoptotic pathway (10), initiated in response to ER stress, such as protein misfolding, protein retention, and disruption of Ca(II) homeostasis in ER (24, 25). It appears that activation of caspase-12 is independent of either death receptor signaling or mitochondrial-targeted apoptotic signals (26), since apoptosis induced by serum deprivation, tumor necrosis factor (TNF), or anti-Fas did not lead to caspase-12 activation. However, ER stress inducers, such as brefeldin A, an inhibitor of ER-Golgi transport, thapsigargin, an inhibitor of Ca(II) ATPase, and A23187, a Ca(II) ionophore, have been shown to activate caspase-12 and lead to apoptosis (10). Calpain has been shown to cleave Bcl-XL and procaspase-12 in vitro and lead to the loss of anti-apoptotic function and caspase-12 activation, respectively (12). However, there are no clear data to show that calpain is the one that catalyzes the activation of procaspase-12 in vivo. Recent studies showed that caspase-12 can be activated by caspase-7, which forms a complex with procaspase-12 (12), and procaspase-12 can also bind to TRAF2, an adaptor protein that couples the plasma membrane receptor to c-Jun NH2-terminal kinase (JNK) activation, and to IRE1,
an ER stress sensor protein kinase (27). Nevertheless, as shown in Fig. 1, calpain inhibitors I and II inhibited caspase-12 activation, and the Ca(II) ionophore A23187 facilitated the cleavage of procaspase-12. Together, these results indicate that Ca(II), in part working through its ability to activate m-calpain (results not shown), plays a major role in caspase-12-mediated apoptosis. This could be accomplished via the cleavage of either procaspase-12, Bcl-xL, or both.

The fact that the p38 MAP kinase inhibitor, SB202190, also exerted strong inhibition of Mn(II)-induced apoptosis indicates the participation of p38 MAP kinase whose activation has been associated with the apoptotic signaling cascade. However, the substrate(s) of this kinase in the regulatory pathway of caspase-12-mediated apoptosis is not known. Nevertheless, both procaspase-12 and one of its binding proteins, TRAF2, have been reported to be phosphorylated by unidentified kinase(s) (27). It is clear that much more work is needed to elucidate this signaling mechanism.

In conclusion, together with the earlier report (17), we show that Mn(II) induced apoptosis in HeLa and NIH3T3 cells via a caspase-12-dependent pathway and independent of the mitochondria-mediated apoptosis. The apoptosis is inhibited by both calpain inhibitors I and II and the p38 MAP kinase inhibitor, SB202190. The results suggest that both calpain and the p38 kinase are involved sequentially without indicating the sequence in activating caspase-12. The role of Mn(II), in part, is to substitute for Ca(II) in activating m-calpain and cause the apoptosis. It should be pointed out that the full-length dsRNAi method works well with NIH3T3 cells, and it is the method of choice for knocking out selected proteins.

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