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Low Micromolar Levels of Hydrogen Peroxide and Proteasome Inhibitors Induce the 60-kDa A170 Stress Protein in Murine Peritoneal Macrophages

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We previously reported cDNA cloning of a novel oxidative stress protein termed A170 from murine macrophages. Further experiments have demonstrated that exposure of the cells to low levels of H_2O_2 produced by glucose/glucose oxidase markedly induced the 60-kDa A170 protein. This result suggests that the level of A170 protein can also be controlled at posttranscriptional levels, because we showed previously that H_2O_2 hardly increased the level of *A170* mRNA. We have found that proteasome inhibitors markedly induced the A170 protein after 2 to 8 h similarly to glucose/glucose oxidase, suggesting rapid degradation of the A170 protein by proteasome under normal conditions. Activation of cellular signaling pathways either by epidermal growth factor, lipopolysaccharide or tumor necrosis factor-α did not enhance the level of the A170 protein. The levels of glucose oxidase-induced A170 protein did not decrease after the addition of cycloheximide. These results suggest that low levels of H_2O_2 may stabilize the A170 protein, allowing it to accumulate within cells.

Macrophages induce various stress proteins to protect themselves upon exposure to reactive oxygen species and to other insulting agents. Induction of stress proteins such as heme oxygenase (1) and MSP23 (2) depend mainly on transcriptional activation. We previously isolated a cDNA encoding a protein of 442 amino acids from murine macrophages by differential screening using diethyl maleate as the stimulant (3). This protein termed A170 has a Zn-finger domain, a PEST domain and several potential phosphorylation sites for serine/threonine kinases, suggesting that it has a regulatory role under oxidative stress. The structure of A170 protein is highly related (roughly 90% identity) to a recently reported 60-62 kDa human lymphocyte protein that has ability to bind the tyrosine kinase $p56^{lck}$ (4,5), a cytokine receptor EBI3 (6), and ubiquitin (7). This structural similarity suggests that the A170 protein could also play a role in signal pathways and could modulate a tyrosine kinase, cytokine signaling, and/or ubiquitin metabolism.

Using an A170-specific antibody, we observed that oxidative stress agents specifically induced a 60-kDa A170 protein in the macrophages *in vitro;* paraquat, diethyl maleate, and cadmium chloride were the most effective inducers. These agents raised the level of *A170* mRNA at most two- to fourfold. However, the expression of the 60 kDa A170 protein did not necessarily depend on the level of *A170* mRNA (3). Moreover the level of the *A170* mRNA significantly decreased in cells during culture in normal medium while the level of the 60 kDa A170 protein significantly increased (3). We previously showed that H_2O_2 or glucose/glucose oxidase that produces H_2O_2 did not significantly enhance the level of *A170* mRNA in the macrophages (3). In this study, we examined effects of H_2O_2 on the expression of the 60-kDa A170 protein. We have found that low levels of H_2O_2 produced by glucose/glucose oxidase markedly induced expression of the A170 protein, suggesting that the induction of A170 protein can also be regulated at post-transcriptional levels. We also provide evidence that the A170 protein is mainly degraded by proteasome at a high rate. Mild oxidative stress may thus specifically retard the degradation of the A170 protein in the macrophages. Low micromolar levels of H_2O_2 may be physiologically important since it is often produced in *in vivo* environment by granulocytes and macrophages during inflammatory processes, and is

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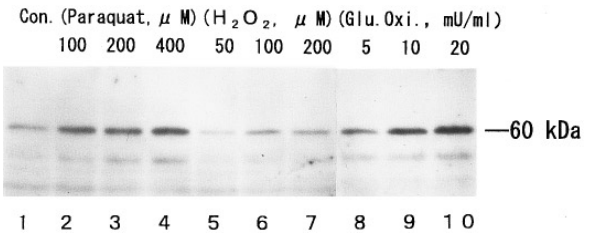

FIG. 1. Immunoblot analysis of the effects of paraquat, H_2O_2 and glucose/glucose oxidase on the level of 60-kDa A170 protein. Lane 1, control; lanes 2-4, 100, 200, and 400 μM paraquat; lanes 5-7, 50, 100, and 200 μM H_2O_2; lanes 8-10, 5, 10, and 20 mU/ml glucose oxidase.

implicated in many pathological situations. Therefore our results suggest that the induction of A170 protein is important in metabolic regulation in the macrophages under mild oxidative stress.

MATERIALS AND METHODS

Culture of macrophages. Peritoneal macrophages were prepared from ddY female mice that had previously been injected with thioglycolate medium and cultured in RPMI1640 medium containing 10% fetal bovine serum as described previously (8).

Reagents. Paraquat, H_2O_2, and glucose oxidase from *Aspergillus niger* were purchased from Wako Pure Chemical Industries. An inhibitor for thiol protease, [L-3-trans-carboxyoxiran-2-carbonyl]-L-leucyl-agmatin (E-64) (9) and three inhibitors for proteasome, carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (MG115) (10, 11), carbobenzoxyl-L-isoleucyl-γ-t-butyl-L-glutamyl-L-alanyl-L-leucinal (PSI) (12, 13),and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) (14, 15) were from Peptide Institute. These inhibitors were dissolved in dimethyl sulfoxide to make 10 or 20 mM stock solutions. Epidermal growth factor was from Mallinckrodt. Lipopolysaccharide was from Difco. Cycloheximide and actinomycin D were from Wako. Tumor necrosis factor (TNF)-α was from Pepro Tech. These agents were added to the culture medium 1 h after cell seeding, and the cells were further incubated for 7 h before harvesting, unless otherwise stated.

Immunoblot analysis. Affinity purified A170-specific antibody from rabbit anti-A170 serum was used as described previously (3). The protein content was determined by BCA protein assay reagent (Pierce). Fifteen or twenty μg of cell proteins were used in each well, and separated by SDS-PAGE. To detect immunoreactive proteins on the transferred membranes, we used horseradish peroxide-conjugated goat anti-rabbit IgG and blotting reagents (ECL, Amersham).

RESULTS

We examined the effects of various doses of H_2O_2 on the level of the A170 protein in murine macrophages. We used the affinity-purified anti-A170 antibody for immunostaining as described previously (3). Single additions of 50, 100, and 200 μM of H_2O_2 to the culture medium did not significantly alter the level of 60-kDa A170 protein after 7 h (Fig. 1, lanes 5-7). Addition of H_2O_2 above 300 μM was toxic, and some cells detached from the dishes after several hours. In contrast to the high doses of H_2O_2, glucose oxidase supplemented at 5 to 20 mU/ml to the culture medium markedly induced the 60-kDa A170 protein (Fig. 1, lanes 8-10) in a dose-dependent manner. These doses of glucose oxidase continuously produce micromolar amounts of H_2O_2 from glucose in the medium during cell culture (16). These effects of glucose oxidase were comparable to that of 100 to 400 μM paraquat used as a standard stress agent (Fig. 1, lanes 2-4).

As we have previously shown, H_2O_2 did not enhance the level of *A170* mRNA in the cells (3). Experiments were performed to investigate post-transcriptional regulation of the level of 60-kDa A170 protein. Since the A170 protein has a PEST motif (3) that suggests a short half-life of the protein, we examined the effects of proteasome inhibitors on the level of A170 protein. PSI and MG115 at 100 μM markedly induced the 60-kDa A170 protein, respectively (Fig. 2, lanes 3 and 4). MG132, another potent inhibitor of proteasome, was more effective than the above two inhibitors in inducing the protein (Fig. 2, lanes 5-7). It was effective at 25 μM in inducing the A170 protein. However, it was cytotoxic at 50-100 μM and the cells gradually detached from the dishes. An inhibitor of thiol proteases, E-64, did not induce the A170 protein at 50 to 200 μM (Fig. 2, lanes 8-10). Dimetyl sulfoxide, used as the solvent for these agents, had no effect on the level of A170 protein (Fig. 2, lane 11). Since the proteasome inhibitors are aldehydes that may have toxic effects on the cells, we also confirmed that formaldehyde at 100 μM had no effect on the level of the protein (not shown). Fig. 3 shows the dependency of the induction of the A170 protein on time. MG132 induced the protein almost linearly 2-8 h after the addition of the agent, after which the level of the protein decreased. Glucose oxidase increased the level of the protein similarly to MG132 for the first 4 h, which peaked at around 6 h and declined following this. After 24 h the level of the protein had returned to the control level (not shown). In the control culture, the level of the protein increased slightly during the first 9 h in culture. These results indicate that the A170 protein is mainly degraded by proteasome.

We next examined the effects of inhibitors of protein

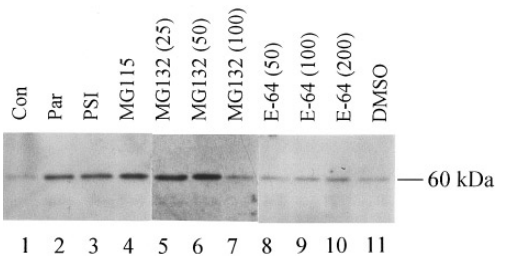

FIG. 2. Effects of protease inhibitors on the level of A170 protein. Lane 1, control; lane 2, 100 μM paraquat; lane 3, 100 μM PSI; lane 4, 100 μM MG115; lane 5-7, 25, 50, and 100 μM MG132; lane 8-10, 50, 100, and 200 μM E-64; lane 11, 1% (v/v) DMSO.

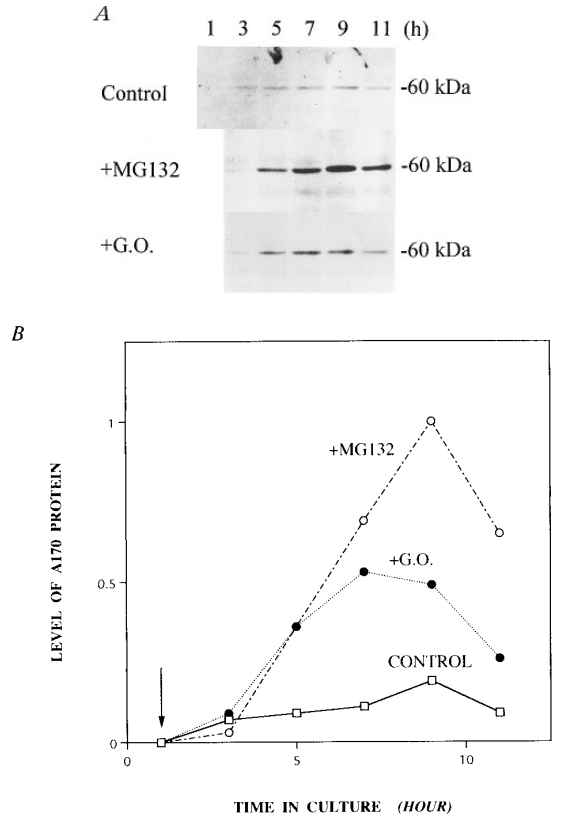

FIG. 3. Time-dependent effects of MG132 and glucose/glucose oxidase on the level of 60-kDa A170 protein. A. Immunoblot analysis. Upper panel, control; middle panel, 50 μM MG132; lower panel, 20 mU/ml glucose oxidase. The time (h) in culture is shown at the top. B. Densitometrical analysis of the immunoblot results. Each agent was added after 1 h in culture (arrow).

and RNA synthesis on the level of expression of the 60 kDa A170 protein. Both cycloheximide and actinomycin D added at the same time with either MG132 or glucose oxidase effectively inhibited the increase in the level of A170 protein expression during culture from 1 to 8 h (Fig.4, lanes 1 to 3). When the cells were first incubated with MG132 or glucose oxidase for 5 h, the addition of cycloheximide apparently stopped the further increase in the level of A170 expression over the next 2 h (Fig.4, lanes 4 to 6). This result may suggest that the glucose oxidase-induced A170 protein is stable. Under the similar experimental conditions, the addition of actinomycin D did not inhibit the further increase in the level of A170 protein in the presence of MG132, but it apparently inhibited the further increase in the protein level in the presence of glucose oxidase over the next 2 h (Fig. 4, lanes 4, 5 and 7).

It has been reported that H_2O_2 activates the mitogen-activated protein kinase (MAPK) family via different signaling pathways (17-19), and that the A170 protein has a potential phosphorylation site for MAPK (3). Therefore, we further examined the effect of agents

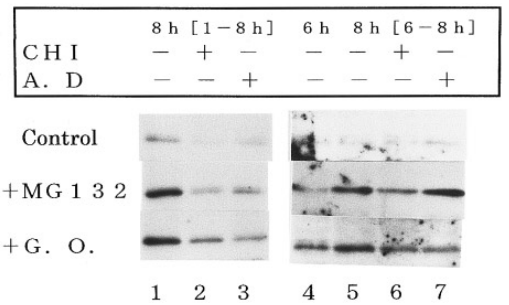

FIG. 4. Effects of cycloheximide and actinomycin D on the level of 60 kDa A170 protein expression analyzed by immunoblotting. One hour after seeding the macrophages, either 25 μM MG132 or 40 mU/ml glucose oxidase (+G.O.) were supplemented to the culture medium and cultured following 5 h (lane 4) or 7 h (other lanes). Additionally, 250 ng/ml cycloheximide (CHI) or 100 ng/ml actinomycin D (A.D) were respectively added to the medium and cultured for 7 h (lanes 2 and 3) or 2 h (lanes 6 and 7) as indicated before harvesting the cells for the analysis.

that respectively activate the MAPK family on the level of A170 protein. We found that the continued exposure of the cells to epidermal growth factor, lipopolysaccharide or TNF-α did not induce the A170 protein (Fig. 5).

DISCUSSION

Using three different protease inhibitors specific to proteasome, we have shown that the A170 protein may possibly be degraded by proteasome (Fig. 2 and 3). This is consistent with our previous finding that the A170 protein has a PEST domain composed of two sequences with remarkably high scores; 26.5 and 16.3 (3). These PEST scores are higher than those found in c-Fos and c-Myc proteins, which have a half-life of about 0.5 h (20). In case of c-Fos protein, the PEST sequence in the C-terminal contributes to the rapid degradation by the ubiquitin plus proteasome system (21). The phosphorylations of c-Fos by a MAPK (ERK) stabilize it (22), but those by multiple kinases accelerate its degradation (23).

We have detected an activity of serine/threonine kinase that potently phosphorylates the A170 protein (unpublished finding). We are also examining whether

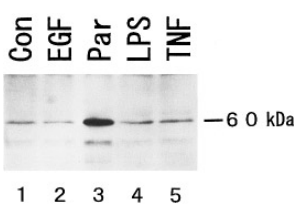

FIG. 5. Effects of various agents that activate cellular signaling pathways on the level of 60-kDa A170 protein. Lane 1, control; lane 2, 0.18 μg/ml epidermal growth factor; lane 3, 100 μM paraquat; lane 4, 10 ng/ml lipopolysaccharide; lane 5, 10 ng/ml TNF-α.

or not the A170 protein can be ubiquitinated as a step of its degradation. The A170 protein therefore provides an example for studying the roles of the PEST-sequence and phosphorylations in the degradation of proteins by proteasome. It seems that the synthesized A170 protein is rapidly degraded when the macrophages are placed under normal conditions or in the peritoneal cavity. It is evidenced by the fact that the *A170* mRNA is expressed (3) but the protein is hardly detected in fresh macrophages prepared from the peritoneal cavity (Fig. 3). We also detected the 2.0 kb *A170* mRNA in seven major murine tissues by northern blotting, but we could not detect the 60-kDa A170 protein in those tissues by immunostaining (not shown).

We have shown here that low levels of H_2O_2 induce the 60-kDa A170 protein in the cells (Fig.1) without significant change in the level of A170 mRNA (3). One of the possibilities to elucidate the above results is that exposure of the cells to low levels of H_2O_2 results in the inhibition of the A170 protein degradation. The 60 kDa A170 protein accumulated in the cells in the presence of glucose oxidase appeared stable. The level of the accumulated protein did not decrease rapidly after the addition of cycloheximide (Fig. 4, lane 6). Apparently H_2O_2 becames less effective than MG132 in raising the level of A170 protein after incubation for 7 h. This may be due to the fact that these cells induce antioxidant proteins such as heme oxygenase (1) and MSP23 (2), and may adapt to the change in environment. MSP23, the major mammalian peroxiredoxin having the thiol-specific antioxidant activity (24), may have an ability to reduce H_2O_2 and alkylhydroperoxides to water and corresponding alcohols similarly to the peroxiredoxin of *Saccharomyces cerevisiae* (25). It is also possible that sulfhydryl-reactive and superoxide-generating agents also work like H_2O_2 to stabilize the A170 protein by an indirect mechanism. The inhibition of the A170 protein degradation should augment the elevation of the A170 protein together with the two- to fourfold enhancement of the level of the *A170* mRNA (3). This hypothesis, however, is contrary to the case in which reactive oxygen species facilitate the degradation of I-κB that results in the activation of the transcriptional factor NF-κB (26, 27). As in the cases of c-Fos and I-κB, further studies of the protein kinase(s) that phosphorylate the A170 protein are needed to elucidate the modulation mechanism of induction of this protein by oxidative stress.

We have shown here that agents which stimulate the MAPK family as part of their effects did not induce the A170 protein (Fig. 5). We performed this experiment because H_2O_2 and other stress-related stimulants such as UV, heat shock, hyperosmolarity, ischaemia/reperfusion injury, TNF-α and lipopolysaccharide induce the MAPK family pathways (28-30). TNF-α and lipopolysaccharide can also induce acute phase antioxidant proteins such as metallothionein and manganese superoxide dismutase (31-33). In conclusion, the induction of the A170 protein appears not to be downstream of the stimulation of the MAPK family and it is induced by non-lethal levels of oxidative stress.

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