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Advances in Brief

Gadd45 and Gadd153 Messenger RNA Levels Are Increased during Hypoxia and after Exposure of Cells to Agents Which Elevate the Levels of the Glucose-regulated Proteins[1]

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Abstract

We have investigated overlapping activation pathways for two families of stress genes that are expressed in cells exposed to hypoxia. The growth arrest and DNA damage (*gadd*) genes are induced by DNA damage and irradiation, and their expression is associated with growth arrest. The glucose-regulated proteins (GRPs) are induced by chemical agents that disrupt protein trafficking in the endoplasmic reticulum such as tunicamycin and A23187 and by hypoxia. Here, we demonstrate that the treatment of NIH-3T3 cells with chemical inducers of GRPs results in increased levels of *gadd45* and *gadd153* mRNA as well as *GRP78* mRNA. In addition, hypoxia was also able to increase *gadd45*, *gadd153*, and *GRP78* mRNA. Therefore the *GRP* and *gadd* genes can be activated by similar stimuli (*e.g.*, hypoxia and chemical inducers). However, the mechanisms leading to increased levels of *GRP78* and *gadd* gene mRNA are different and may involve distinct protein kinases. Increased expression of GRPs after treatment with chemical inducers is sensitive to cycloheximide and the protein kinase inhibitors genistein, 2-aminopurine, and H7, whereas the increase in *gadd* gene mRNA could be blocked by the protein kinase inhibitors H7 and 2-aminopurine but not by genistein or cycloheximide. GRP78 induction occurs by a pathway that requires protein synthesis and is sensitive to genistein, H7, and 2-aminopurine, whereas *gadd* gene induction is independent of protein synthesis and is inhibited by H7 and 2-aminopurine only.

Introduction

The *gadd*[3] genes are induced by growth arrest, by DNA damage, or by X-ray or UV irradiation (1–3). One member of the *gadd* gene family, *gadd45*, is strongly induced by low doses (2 Gy) of X-ray irradiation by a mechanism which does not involve activation of protein kinase C (4). One of the main effects of the radiation of cells is to induce growth arrest, with cells being blocked in G_2 (6, 7). This block is thought to allow cells time to repair DNA damage before proceeding to cell division (6, 7). Cells which are forced to proceed past this block without allowing time for DNA repair (by treating the cells with caffeine) exhibit reduced cell survival and DNA fragmentation (7). In yeast, the *rad9* gene is thought to be responsible for blocking cells in G_2 after irradiation (6). The *gadd* genes may encode mammalian proteins with functions similar to that of the *rad9* gene, although they have no significant sequence homology to the *rad9* gene (3, 4). *gadd45* has 60% homology to *MyD118*, a gene induced in murine myeloblastic leukemia cells during terminal differentiation and growth arrest (5). Thus the induction of the *gadd* genes by radiation and/or DNA-damaging agents may be required to growth arrest the cells and allow time for DNA repair. We were interested in how variations in the environment of the cell might affect the expression of the *gadd* genes. Hypoxia has been shown, using tissue culture systems, to confer radioresistance on cells (8), and hypoxic cells have been detected in tumors (9). However, there has been little study on how environmental conditions such as hypoxia may affect gene expression and how this may affect the response of cells to irradiation. The best-studied gene family whose transcription is altered by hypoxia is the GRPs. GRPs are major protein constituents of the ER (10–13). GRP78 functions as a molecular chaperone in the ER, where it participates in the folding of nascent proteins and the formation of oligomeric proteins (14). When cells are deprived of glucose (12), become hypoxic (11), or are treated with stress compounds that interfere with ER function (*e.g.*, tunicamycin, A23187, thapsigargin) (12, 13, 15), the transcription of the GRPs is greatly increased. These stress agents may all act by inhibiting the glycosylation of proteins in the ER, which results in the retention of proteins within the lumen of the ER (10–14). This accumulation of proteins may be the stimulus for enhanced GRP transcription. Here, we show that both hypoxia and chemical inducers of GRP expression can cause increased expression of the *gadd* genes. However, the induction of the *gadd* genes apparently occurs by a different pathway to the induction of GRPs, since the two gene families showed differential sensitivities to protein synthesis inhibitors (cycloheximide) and protein kinase inhibitors (genistein, 2-aminopurine, H7).

Materials and Methods

Cell Culture, Inhibitor Treatment, and Hypoxia. NIH-3T3 cells were grown to about 90% confluency in Dulbecco's modified Eagle's medium containing 10% bovine calf serum. Inhibitors (obtained from Sigma Chemical Co., St. Louis, MO) were added at time zero and remained in the medium throughout the experiment unless otherwise stated. Cells were exposed to hypoxia as follows. NIH-3T3 cells were overlain with 10 ml of fresh Dulbecco's modified Eagle's medium/10% serum (glucose = 4.5 g/dm^{-3}), sealed in air-tight chambers, and maintained at 37°C throughout the subsequent manipulations. The 95% air/5% CO_2 medium was removed by flushing the chambers with 95% nitrogen/5% CO_2, after which the concentration of oxygen was calculated to be 67 ppm (16). During hypoxia, approximately 20% of the available glucose was used after 20 h, and the pH of the medium remained close to 7.4 throughout the experiment. Cell viability was monitored with trypan blue; essentially all cells (>96%) excluded the dye at the end of the treatment, except for hypoxic cells incubated in either H7 or 2-aminopurine, 30% of which stained at the end of the incubation. For this reason, the treatment of hypoxic cells with H7 or 2-aminopurine was not studied (see text).

Northern Blotting. After the treatment of cells with inducers, cells were washed twice in phosphate-buffered saline, and total cellular RNA was prepared using the guanidinium isothiocyanate method (17). RNA (15 μg) was loaded and separated on 1% agarose/2.2 M formaldehyde

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[3] The abbreviations used are: gadd, growth arrest and DNA damage; GRP, glucose-regulated proteins; ER, endoplasmic reticulum.

gels. Equal RNA loading was confirmed by measuring the concentration of RNA (absorbance, 260 nm) and by staining the agarose gels with ethidium bromide and assessing the intensity of the 18S and 28S rRNA bands. After running the gels, RNA was transferred to Genescreen (Du Pont-New England Nuclear) by capillary blotting and prehybridized (18). *GRP78* mRNA was detected with a 750-base pair fragment from the 3′ end of the *GRP78* gene. *Gadd45* and *gadd153* mRNAs were detected using fragments of the gene, kindly provided by Dr. Fornace (3). Hybridization and washing (in 1 × standard saline citrate/0.5% sodium dodecyl sulfate; 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) were carried out as previously described (18).

Results and Discussion

A23187 and tunicamycin have been shown to induce GRP expression in a number of cell lines (11–13). We have recently shown that thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase (15), is also a strong inducer of the *GRP* genes and, since it is less toxic than A23187 (15), we have chosen to use it in this study. Fig. 1 shows the effect of a number of GRP inducers on the levels of *gadd45* (Fig. 1, *top*), *gadd153* (Fig. 1, *middle*), and *GRP78* (Fig. 1, *bottom*) mRNA. Fig. 1 (*Lanes 1–4*) shows that cells incubated with A23187, tunicamycin, or thapsigargin all show strong increases in the level of *gadd45*, *gadd153*, and *GRP78* mRNA. In cells exposed to hypoxia (Fig. 1, *Lane 6*), increased levels of *gadd* and *GRP78* mRNA can also be seen compared to controls (Fig. 1, *lane 5*), although the levels of induction are less than with chemical inducers. Fig. 1 demonstrates that both hypoxia and chemical inducers can cause

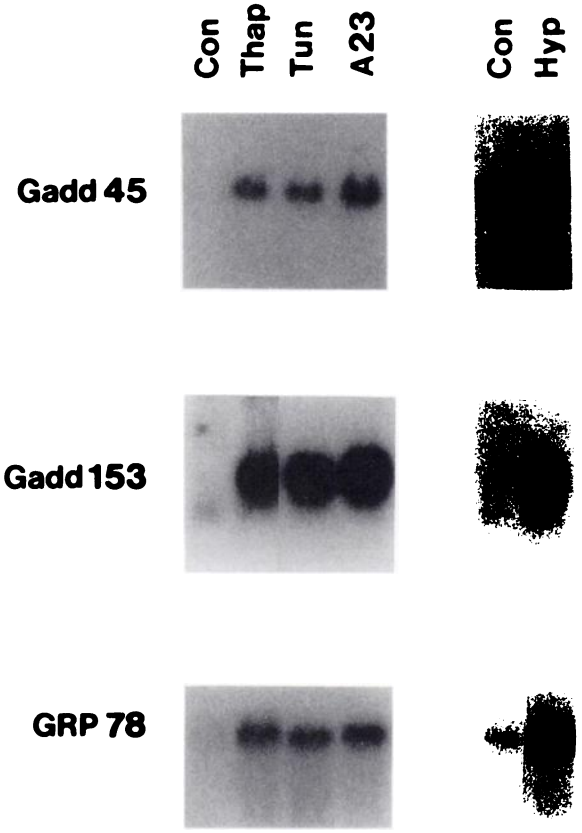

Fig. 1. Accumulation of *gadd45*, *gadd153*, and *GRP78* mRNA after thapsigargin and hypoxia treatment. NIH-3T3 cells were either untreated (*Con*) or treated with thapsigargin (*Thap*; 2 μM), tunicamycin (*Tun*; 10 μg/ml), or A23187 (A23; 2 μM) for 8 h. Cells were then washed free of medium, and RNA was prepared as described in "Materials and Methods." Hypoxia (*Hyp*) exposure was achieved as described in "Materials and Methods." RNA was separated, transferred to nylon membranes, and hybridized with a gadd45 (*top*), gadd153 (*middle*), or GRP78 (*bottom*) probe.

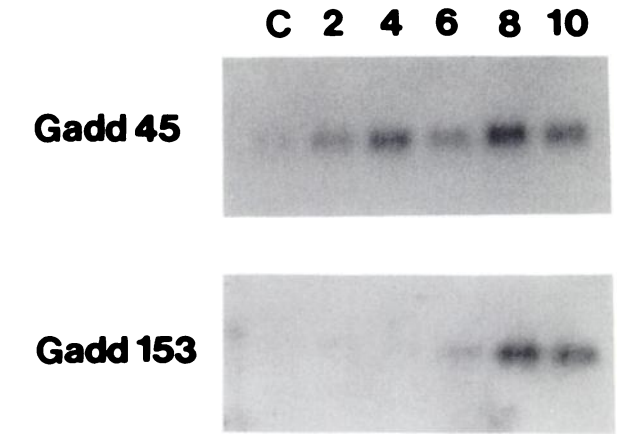

Fig. 2. Time course of *gadd45* and *gadd153* mRNA accumulation after thapsigargin treatment. NIH-3T3 cells were either untreated (*Lane C*) or incubated with thapsigargin (2 μM) for 2, 4, 6, 8, or 10 h as indicated. RNA was extracted, and the levels of *gadd45* mRNA (*top*) or gadd153 mRNA (*bottom*) were assessed by Northern blotting.

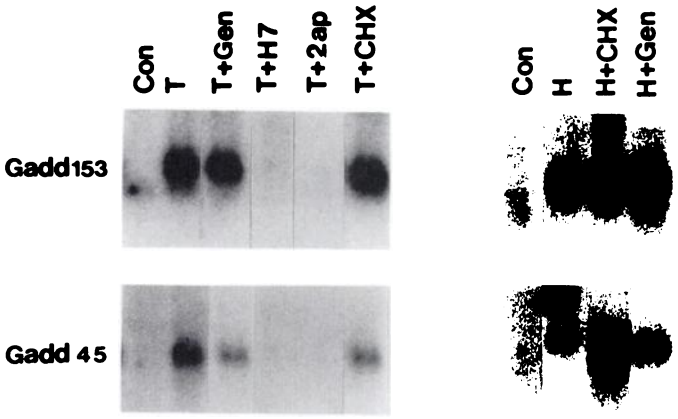

Fig. 3. Effect of protein kinase inhibitors and cycloheximide on the accumulation of *gadd* gene mRNA after thapsigargin or hypoxia treatment. *Left*, untreated (*Con*) or incubated with thapsigargin (*T*; 2 μM) in the presence of genistein (*T + Gen*; 140 μM), H7 (*T + H7*; 60 μM), 2-aminopurine (*T + 2ap*; 10 mM), or cycloheximide (*T + CHX*; 10 μg/ml) for 6 h. RNA was then prepared, and Northern blotting was carried out to identify *gadd153* mRNA (*top left*) or *gadd45* mRNA (*bottom left*). *Right*, cells either maintained in air (*Con*) or made hypoxic (*H*) in the presence of cycloheximide (*H + CHX*; 10 μg/ml) or genistein (*H + Gen*; 140 μM). RNA was then prepared, and Northern blotting was carried out to identify *gadd153* mRNA (*top right*) or *gadd45* mRNA (*bottom right*).

accumulation of *gadd45*, and *gadd153*, and *GRP78* mRNA.

In Fig. 2, cells were stimulated with thapsigargin, and the RNA was extracted at the indicated times. Both *gadd*45 and *gadd*153 can be detected as early as 2 h after stimulation, with accumulation continuing for at least 8 h, peaking by 10 h. This time course exactly mirrors the induction of GRP78 after thapsigargin addition (15) and demonstrates that the elevated levels of *gadd* gene mRNA is not a transient response to thapsigargin but represents long-term (10 h) accumulation.

In Fig. 3, the signaling pathways involved in the activation of the *gadd* genes were studied. Cycloheximide was used to inhibit protein synthesis, and the general protein kinase inhibitors H7 (which can antagonize protein kinase C and other kinases), 2-aminopurine (which has broad specificity toward ser/thr kinases), and genistein (specific for tyr protein kinases) (18) were used to block phosphorylation. In a previous study, we demonstrated that the accumulation of *GRP78* mRNA after thapsigargin treatment could be inhibited by both genistein and cycloheximide (18). The experiment in Fig. 3 was carried out

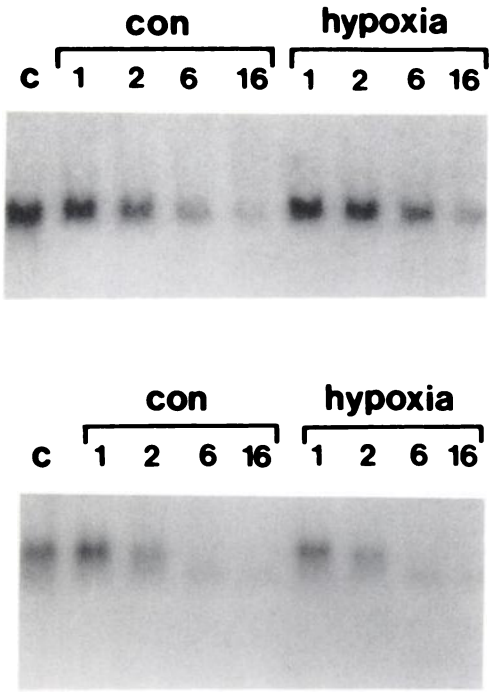

Fig. 4. Effect of hypoxia on the half-life of *gadd45* and *gadd153* mRNA. Cells were exposed to thapsigargin (2 μM) for 4 h to allow accumulation of *gadd* gene mRNA. Cells were then washed once in fresh medium, actinomycin D (10 μg/ml) was added, and the cells were either allowed to continue incubating in air (*con*) or were made hypoxic (*hypoxia*). RNA from one set of cells was extracted immediately after the addition of actinomycin D (*Lane C*). One 2, 6, or 16 h later, RNA was extracted, and the levels of *gadd45* mRNA (*top*) and *gadd153* mRNA (*bottom*) were assessed by Northern blotting.

to determine if the changes in *gadd* gene mRNA levels were also sensitive to genistein and cycloheximide. In Fig. 3, cells were stimulated with thapsigargin in the absence or presence of the indicated inhibitors (Fig. 3, *left*). Induction of *gadd*45 and *gadd*153 was greatly decreased in the presence of H7 and 2-aminopurine (Fig. 3, *Lanes T + H7* and *T + 2ap*). This indicates that protein kinases may play an important role in the induction of the *gadd* genes. H7 is known to inhibit protein kinase C as well as other kinases, but since the *gadd* genes are not induced by phorbol esters, which are powerful agonists of protein kinase C (4),[4] it is likely that H7 is inhibiting some other, as yet unidentified protein kinase. Genistein had no effect on *gadd153* mRNA levels (Fig. 3, *Lane T + Gen*) and caused only a small reduction in *gadd45* mRNA levels. In contrast, we have previously shown that GRP78 induction by thapsigargin is blocked by genistein (15) as well as by 2-aminopurine and H7.[4] This suggests that the protein kinases involved in the induction of GRPs after thapsigargin treatment are distinct from those involved in *gadd* gene expression. This difference between the induction of the *gadd* genes and *GRP* genes by thapsigargin was further explored using the protein synthesis inhibitor cycloheximide. The induction of *gadd153* mRNA by thapsigargin was not blocked by cycloheximide (Fig. 3, *Lane T + CHX*), and accumulation of gadd45 was only partly blocked. This indicates that increased *gadd* gene mRNA does not require the synthesis of new proteins but can occur by the activation of existing systems, whereas increased GRPs require new protein synthesis (15). Thus although both GRP and *gadd* gene mRNA are increased by thapsigargin, activation of these genes occurs by two separate pathways.

[4] B. Price, unpublished observation.

The effect of protein kinase inhibitors and protein synthesis on the increases in *gadd* gene mRNA during hypoxia were also studied. Because 2-aminopurine and H7 caused significant cell death when added to hypoxic cells (approximately 30% of cells were stained with trypan blue after incubation under hypoxic conditions with these inhibitors; see "Materials and Methods"), we were unable to determine if these inhibitors could affect the induction of *gadd* gene mRNA by hypoxia (data not shown). In Fig. 3 (*top right*), hypoxic cells (Fig. 3, *Lane H*) show increased levels of *gadd153* mRNA, and this accumulation of *gadd153* mRNA was unaltered by either cycloheximide (Fig. 3, *Lane H + CHX*) or genistein (Fig. 3, *Lane H + Gen*). Hypoxia increases *gadd45* mRNA (Fig. 3, *bottom*, *Lane H*), and the addition of genistein (Fig. 3, *Lane H + Gen*) did not alter this, in contrast to the slight inhibitory effects of genistein on *gadd45* induction after thapsigargin treatment (Fig. 3, *left*). Treatment of hypoxic cells with cycloheximide enhanced the accumulation of *gadd45* mRNA compared to hypoxia alone (*Lane 3*, *bottom right*). This suggests that there are some differences in the mechanism of induction after hypoxic exposure compared to treatment with thapsigargin.

Cycloheximide has been shown to cause the superinduction of a number of rapidly turned over mRNAs by stabilizing the message (19). To assess whether any change in mRNA stability occurred during hypoxia, the stability of the *gadd45* and *gadd153* mRNAs was determined. Cells were stimulated with thapsigargin and allowed to accumulate mRNA for 4 h before the addition of actinomycin D. The rate of decrease in mRNA levels was then determined over the next 16 h (Fig. 4) in either air/CO_2 (Fig. 4, *con*) or N_2/CO_2 (Fig. 4, *hypoxia*) atmosphere. Both *gadd* genes had half-lives of approximately 60 min under aerobic conditions (Fig. 4, *con*). During hypoxia, the half-life of *gadd153* mRNA was unaltered, while the half-life of *gadd45* mRNA was increased slightly from 65 min to approximately 95 min (Fig. 4, *hypoxia*). Because the levels of gadd45 and gadd153 are almost undetectable in untreated NIH-3T3 cells, it was not possible to determine the stability of the mRNAs in unstimulated cells.

The data indicate that cells exposed to either hypoxia or GRP inducers express both the *gadd* genes and *GRP78*, indicating some overlap in their activation pathways. However, the function of these two gene families is quite different, with *gadd* genes involved in growth arrest and *GRP* genes in protein folding (1, 14). It is possible that stress induces two types of response. First, cells become growth arrested (due to *gadd* gene expression) to allow the second step, the production of stress proteins (*e.g.*, GRPs) and damage repair to take place. Other gene products are likely to be involved.

The induction of these two gene families appears to occur by distinct mechanisms. This inference is based on the observation that the accumulation of *GRP78* mRNA after thapsigargin treatment is sensitive to genistein, 2-aminopurine, H7, and cycloheximide (15), whereas accumulation of *gadd* gene mRNA was sensitive to 2-aminopurine and H7, but not cycloheximide or genistein (Fig. 3). Accumulation of *GRP78* mRNA requires new protein synthesis (blocked by cycloheximide) (15), whereas increases in *gadd* gene mRNA do not. This suggests that GRP transcription may require the synthesis of new protein factors involved in regulating gene transcription, whereas *gadd* gene transcription results from the activation of preexisting factors. A further difference between these two gene families is detailed by their differential sensitivity to protein kinase inhibitors. Accumulation of *GRP78* mRNA is sensitive to H7, 2-amino-

purine (specific for ser/thr kinases), and genistein (specific for tyrosine kinases), while *gadd* gene expression is sensitive to only H7 and 2-aminopurine. Genistein is a well-established inhibitor of tyrosine kinases (15, 18), and the differential sensitivity of GRPs and *gadd* genes to it may indicate that *GRP* expression involves a tyrosine phosphorylation event which is not required for *gadd* gene expression. It has previously been shown that irradiation of cells increases *gadd45* mRNA levels and that this is sensitive to H7 (4), which agrees with the results shown here. As we have shown before, hypoxia may activate a pathway separate from those of chemical inducers, since the hypoxic induction of GRPs is not blocked by cycloheximide (15). During hypoxia, the half-life of *gadd45* mRNA is slightly increased, but the half-life of *gadd153* mRNA is unaffected. It is possible that some control over the levels of *gadd* genes mRNA is exerted at the level of mRNA stability (1–4), especially during hypoxia.

These results suggest that a range of stimuli, including hypoxia, GRP inducers, and irradiation, can activate diverse signaling systems within the cell, some of which converge on similar gene targets such as *gadd* genes or GRPs, and enhance their transcription. However, the route by which these signals are sent may differ, depending on the stimulus encountered, and may indicate that there are other genes involved which have yet to be identified.

We have shown that *gadd* gene mRNA can be increased by compounds that cause the induction of the GRPs in fibroblast cell lines and that the exposure of cells to hypoxia will also increase the *gadd* genes. This has a number of important consequences, since it means that cells exposed to hypoxia, whether *in vitro* or *in vivo*, can potentially express both GRPs and *gadd* genes. *gadd* genes are also induced in response to irradiation and DNA damage (1) and may have a role in mediating the G_2 arrest of cells which occurs after irradiation (1–4). Their expression in hypoxic cells may make some contribution to the inherent radioresistance of hypoxic cells seen *in vitro* (8, 9). It is also of interest to note that these data show that some radioinduced genes (*i.e.*, *gadd* genes) may also be activated by hypoxia. In conclusion, this study has outlined a number of steps in the induction of both the *gadd* genes and GRPs under environmental stress conditions and should allow further studies to help define the mechanisms of the activation of these genes.

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