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Induction of glucose-regulated proteins during anaerobic exposure and of heat-shock proteins after reoxygenation

(stress proteins/Chinese hamster cells/hypoxia)

JAMES J. SCIANDRA, JOHN R. SUBJECK*, AND CHRISTINE S. HUGHES

Division of Radiation Biology, Roswell Park Memorial Institute, Buffalo, NY 14263

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ABSTRACT **In this report we examine the effects of chronic anaerobic exposure and subsequent reoxygenation on protein synthesis patterns in Chinese hamster ovary cells. It is observed by two-dimensional gel electrophoresis (isoelectric focusing/NaDodSO$_4$/PAGE) that the transition from an atmospheric environment to an anaerobic state transiently induces the major heat-shock proteins (at 68 and 89 kDa). As the period of anaerobiosis increases, this heat-shock induction disappears and a new set of proteins (at 76 and 97 kDa) is induced. By two-dimensional gel electrophoresis and partial proteolytic mapping, these new proteins, which are induced by anaerobic exposures exceeding 12 hr, are identical to 76 and 97 kDa (p76 and p97, respectively) proteins induced by extended periods of glucose deprivation (>14 hr) when oxygen is present. Furthermore, the induction of these proteins under anoxia occurs in the presence of glucose, and increasing the glucose content of the starting media does not affect the induction. When anaerobic p76 and p97 induced cells are returned to atmospheric oxygen, p76 and p97 are repressed, while the heat-shock proteins are again transiently induced. This work further suggests the importance of deprivation and release environments in controlling the expression of these two stress protein systems. It is suggested that their natural expression may be determined by comparable circumstances.**

In response to certain adverse environments, cells are known to express sets of proteins. While there have been several suggestions regarding the function of the induced proteins, they may be responsible for adjusting a cell to the new environment and, perhaps, in temporarily maintaining it once there. Hyperthermia and glucose deprivation are two well-recognized conditions that can elicit specific protein systems. Glucose deprivation has been shown to result in the induction of two major proteins of ≈97 and 76 kDa (1, 2), while hyperthermia is known to induce proteins of 68–70, 89, and 110 kDa (3, 4). Anoxia is also an insult that has been shown to induce specific anaerobic shock proteins (5), and early studies in lower organisms have suggested that release from anoxia can elicit a heat-shock response (ref. 22; reviewed in ref. 6). Understanding the response of cells to temporary periods of anoxia has important applications in several areas of biology. In this report, we examine the effects of a transition to and release from a chronic anaerobic exposure on protein synthesis in Chinese hamster ovary cells.

METHODS

Glucose and Oxygen Deprivation. Chinese hamster ovary cells (CHO cells) were grown in Ham's F-10 medium containing 15% newborn calf serum (GIBCO). Cells (5 ml) were seeded at a density of 150,000 cells per ml to T25 flasks (Corning) and were used for experiments in a subconfluent state ≈48 hr later. To achieve glucose deprivation, 15% serum was added to F-10 medium prepared without glucose, resulting in a partially glucose deficient broth. During incubation, cells used the remaining glucose after 20 hr, as determined using a Sigma glucose colorimetric assay kit. Glucose-deprived cells were harvested after an additional 24 hr of incubation. To achieve anoxia, cultures in full medium (or in full medium containing 50% additional glucose) were placed in a sealed Brewer jar (Baltimore Biological Laboratory, Microbiology Systems) and anaerobiosis was initiated by using a hydrogen generator in a 4%–7% carbon dioxide atmosphere as described (7, 8). The oxygen concentration in the jar is decreased to <0.4% in 100 min (8), and the concentration of oxygen at cell depth in a nonagitated solution has been calculated (9) to be within 1% of the environmental value within 30 min. The formation of water vapor from hydrogen and oxygen caused a brief (15 min) temperature increase to 38.6°C in the culture medium soon after initiation of anaerobiosis. This increase is insufficient to elicit a heat-shock response. Anoxia was verified using a methylene blue indicator solution (8). This solution becomes colorless (due to the absence of oxygen) 5–6 hr after the initiation of anaerobiosis. In these experiments, medium was changed both 24 hr prior to and immediately prior to the initiation of anaerobiosis. This ensured a constant starting glucose concentration (1 g/liter) in each experiment. Under anoxia, glucose is used more rapidly than when oxygen is present, and in all studies a starting glucose concentration of 1.5 g/liter was also used (by adding 0.5 g of additional glucose per liter) and results were replicated under these conditions. While the glucose was close to depletion at 24 hr when a 1× glucose concentration was used, an approximate concentration of 0.5 g/liter was still present at 24 hr with 1.5× starting concentration. The anoxia and reoxygenation results presented here reflect data from several independent experiments.

Gel Electrophoresis. For both glucose and oxygen deprivation, cells were pulse-labeled for 1 hr with [^{35}S]methionine (Amersham; 1400 Ci/mmol; 1 Ci = 37 GBq) either before or after the indicated periods of glucose deprivation or anoxia. Under anoxia, 500 μl of de-aerated medium containing label was added (via a catheter) and flushed with a 1-ml volume of nitrogen. Studies revealed that the patterns of protein synthesis immediately upon reoxygenation (or restoration of glucose) were identical to those under anoxia (or glucose deprivation). One-dimensional NaDodSO$_4$/PAGE was carried out as described (10–12). Isoelectric focusing/NaDodSO$_4$/PAGE was carried out as described (13) and modified to exclude Nonidet P-40 from the urea sample buffer (14). This focuses proteins with isoelectric points in the pH range of 4 to 7. Gels were dried and exposed to Kodak XAR film for 24 hr. Equal masses of protein were loaded using the Bio-Rad protein determination assay. Molecular sizes are estimated from Bio-Rad low and high molecular weight standards. Pro-

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Abbreviation: CHO, Chinese hamster ovary.

*To whom correspondence should be addressed.

teolytic mapping of the induced proteins was carried out by cutting radioactive spots from two-dimensional gels followed by partial digestion with *Staphylococcus aureus* V8 protease as described (15). A 96-hr exposure of the digests to Kodak XAR film was used.

RESULTS

The pattern of protein synthesis in CHO cells after the initiation of anaerobiosis is shown in Fig. 1. At 4 hr into the experiment (at a time when the indicator solution is not fully reduced) an induction in the synthesis of a protein of 68 kDa above control levels is visible. This protein appears similar to the major inducible protein of this relative mobility, which results from the application of heat stress. Fig. 2 *a* and *b* presents a two-dimensional analysis of the pattern of protein synthesis in control cells (*a*) and 4 hr into the anaerobic experiment (*b*). By this analysis, the 68-kDa protein is indicated as well as an 89-kDa protein. In Fig. 2*c*, one sees the effect of the application of a 45°C 5-min heat shock on CHO cells. The arrows in this frame indicate the well-known major heat-shock proteins at 68 and 89 kDa (pI values of 5.6 and 5.2, respectively), which are seen to be identical to the proteins indicated in Fig. 2*b*. The 110-kDa heat-inducible protein is not strongly induced by transition to anoxia.

As the period of anaerobiosis increases, the increased synthesis rates of these two heat-shock proteins is not maintained and is decreased to normal (or below normal) levels. During these longer anaerobic exposures (>12 hr), two new proteins are observed to be induced with molecular sizes of 76 and 97 kDa. The 76-kDa protein is the more sensitive of the two proteins to induction by anoxia, and only a slight increase in the intensity of the 97-kDa protein is visible in Fig. 1. These proteins remain induced until the termination of the experiment at 24 hr. Two-dimensional gel electrophoresis of the proteins synthesized after 16 hr of anoxia are presented in Fig. 3. The major proteins induced by this lengthy period of anoxia are indicated. The induction of the

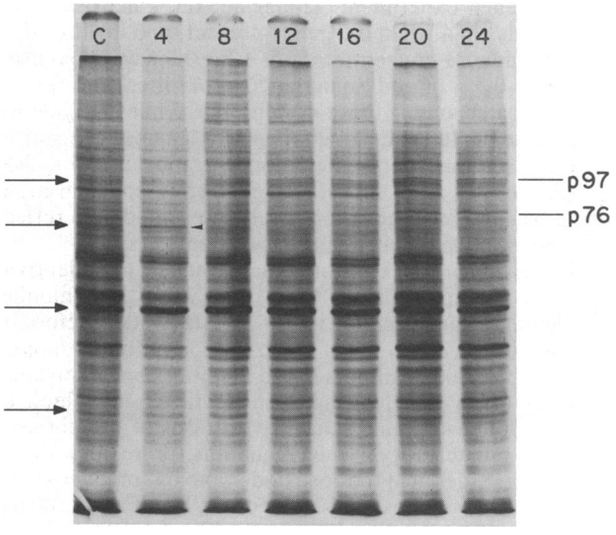

FIG. 1. Autoradiogram of the protein synthesis pattern in CHO cells after various intervals of anaerobic incubation in a Brewer jar. Hours of incubation after initiation of the anaerobic exposure are indicated above the lanes. At 4 hr, a transient induction of a 68-kDa protein is observed (arrowhead). At later times, a gradual induction of 76- and 97-kDa proteins is observed (p76 and p97). Molecular size standards indicated by arrows (top to bottom): phosphorylase B, 92,500 kDa; bovine serum albumin, 66,200; ovalbumin, 43,000; and carbonic anhydrase, 31,000. Equal masses of protein were loaded in each lane by using the Bio-Rad protein determination assay. C, control (no incubation).

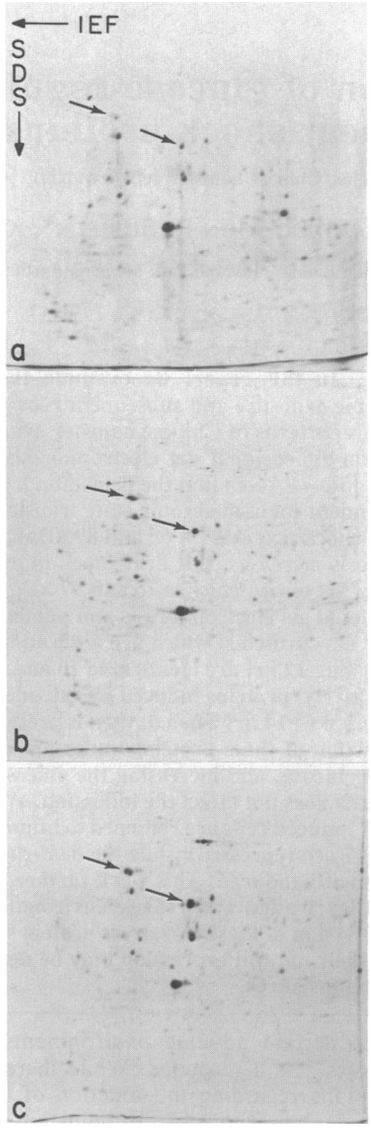

FIG. 2. Two-dimensional gel electrophoresis showing the induction of 89- and 68-kDa proteins (arrows) in CHO cells as a result of transition to anoxia (*b*) and heat shock (*c*). (*a*) Control pattern of protein synthesis; (*b*) 4 hr into the anaerobic exposure; (*c*) 45°C and 5 min of heat shock (plus a 4 hr incubation at 37°C). Equal masses of protein were loaded. Autoradiograms only are shown. IEF, isoelectric focusing; SDS, $NaDodSO_4$.

76-kDa protein is clearly evident, and by this approach one can also clearly distinguish the induction of the 97-kDa protein. For comparison, Fig. 4 shows the pattern of protein synthesis in CHO cells before and after 24 hr of glucose deprivation in the presence of atmospheric oxygen. The arrows in this figure distinguish two inducible proteins of 97 and 76 kDa (pI values of 5.0 and 5.2, respectively), which are the major glucose regulated proteins. It is evident that the two major proteins induced by anaerobic exposure (in the presence of ample glucose) or as a result of glucose deprivation (in a culture dish exposed to atmospheric oxygen) appear to be identical by these criteria. While these proteins are prominant and well recognized from several earlier glucose deprivation studies (1, 2, 16), it should be noted that certain other proteins are also induced in these environments.

To further substantiate this observation, the 76- and 97-kDa proteins (designated p76 and p97) were cut from two-dimensional gels of glucose-deprived and anaerobic-induced cells, similar to those shown in Figs. 3*b* and 4*b*, and subject-

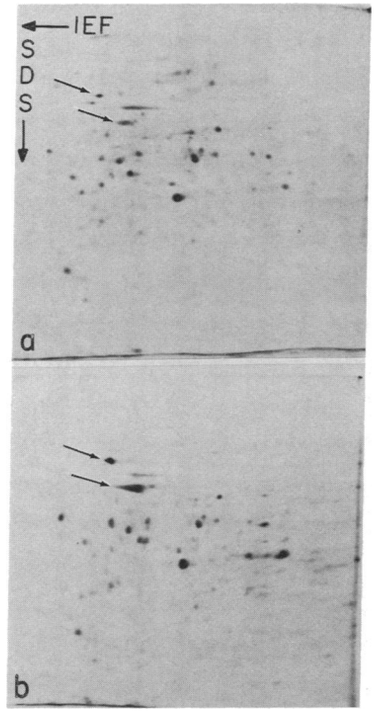

FIG. 3. Two-dimensional gel electrophoresis of the induction of p76 and p97 (arrows) as a result of chronic anaerobic exposure. Protein synthesis pattern of control CHO cells (*a*) and of cells after a 16-hr anaerobic exposure (*b*) are shown. Glucose is present in the medium after 16 hr of anoxia, and increasing the glucose content of the starting medium by 50% did not affect the induction of these proteins. Autoradiograms only are shown and equal masses of protein were loaded. IEF, isoelectric focusing; SDS, $NaDodSO_4$.

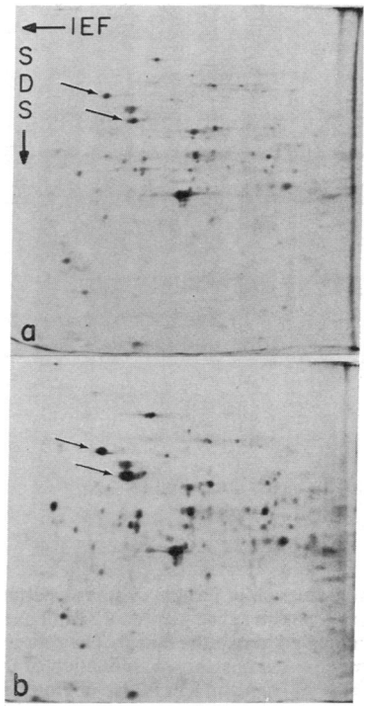

FIG. 4. Two-dimensional gel electrophoresis of the induction of p76 and p97 (arrows) as a result of glucose deprivation. Protein synthesis pattern of control CHO cells (*a*) and cells 24 hr after glucose was depleted from the medium (*b*) are shown. The two induced proteins indicated in *b* appear identical to the proteins induced under anoxia. Autoradiograms only are presented and equal masses of protein were loaded. IEF, isoelectric focusing; SDS, $NaDodSO_4$.

ed to limited proteolysis. Fig. 5 presents autoradiograms of the peptide patterns of these inducible proteins. Comparison of the patterns indicates that p76 and p97, which are induced by glucose deprivation, are identical to p76 and p97, respectively, induced by chronic anoxia. These maps further indicate that p76 and p97 are not homologous with one another. It should be further added that induction of p76 and p97 under anoxia was, in general, less significant than induction via glucose deprivation.

After a 24-hr period in the Brewer jar, the chambers were opened and the cultures were returned to standard incubator conditions. The pattern of protein synthesis as a function of time after re-aeration is presented in Fig. 6. It is seen here that p76 and p97, induced during the chronic anaerobic exposure, are initially synthesized at an increased level relative to their control level of synthesis. Re-aeration, however, is seen to eventually repress the synthesis of p76, while p97 is repressed more slowly. Concurrent with this repression, the induction of 68- and 89-kDa proteins is observed. In Fig. 7*b*, one sees a two-dimensional analysis of the pattern of protein synthesis 8 hr after release from anoxia. An induction of proteins at 68 and 89 kDa is visible above their level of synthesis in CHO cells under aerated and control conditions (Fig. 7*a*). These two proteins have already been identified (in Fig. 2) as the major heat-shock proteins at these molecular sizes. Therefore, it is evident that the proteins induced by release from anoxia appear to be identical to the major proteins that are well recognized to result from the application of heat stress. It is seen from Fig. 6 that the induction of these heat-shock proteins is approximately concurrent with the repression p76. As the cells are maintained under aerated conditions, these heat-shock proteins are eventually repressed, and the control pattern of protein synthesis is again restored within 24 hr after aeration.

During this entire period of anoxia and release from anoxia, glucose was not totally exhausted from the medium and increasing the glucose content in the starting medium by 50% (to a final concentration of 1.5 g/liter) did not significantly alter the observations or kinetics of data reported here. However, re-aeration of cells after 10 hr of anoxia did not result in heat-shock protein induction, suggesting a prerequi-

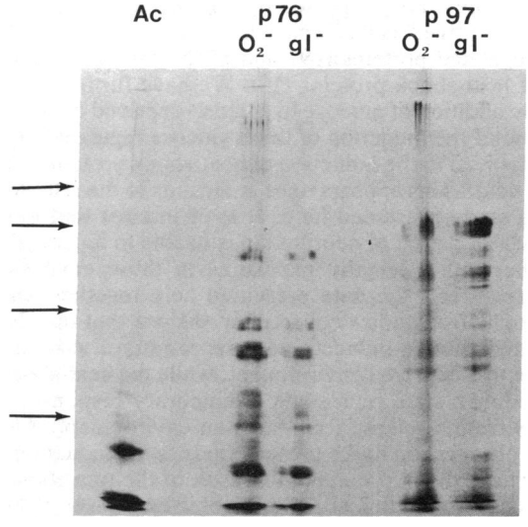

FIG. 5. Proteolytic mapping of p76 and p97 induced by glucose deprivation (gl$^-$) or oxygen deprivation (O_2^-). Radioactive spots were cut from induced two-dimensional gels, similar to those in Figs. 3*b* and 4*b*, and were partially digested with *S. aureus* V8 protease. Ac, actin. Autoradiogram is shown. Molecular size standards indicated by arrows are the same as in Fig. 1.

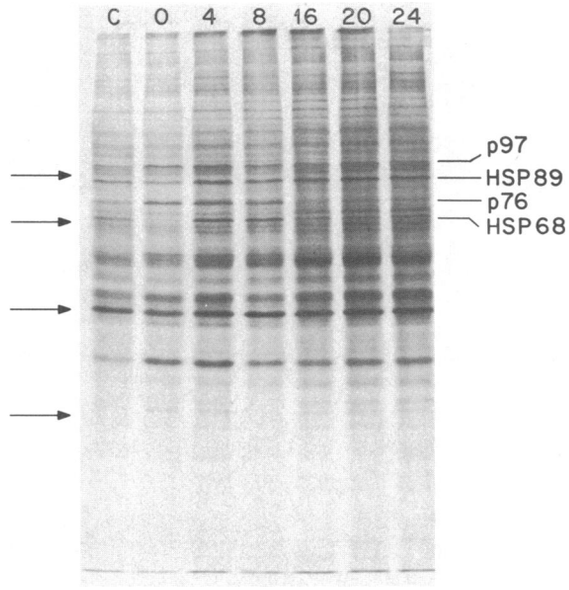

FIG. 6. Autoradiogram of protein synthesis pattern after various intervals of reoxygenation subsequent to a 24-hr anaerobic exposure (time in hours indicated above the lanes). The repression of p76 and p97, induced under anoxia, is shown. Induction of the 68-kDa heat-shock protein (HSP 68) at 4 and 8 hr is also obvious. HSP 89, 89-kDa heat-shock protein. Molecular size standards indicated by arrows are the same as in Fig. 1. Equal masses of protein were loaded. A starting concentration of 1.5 g of glucose per liter was used, and glucose was not depleted during the entire period of anoxia and subsequent release. C, control.

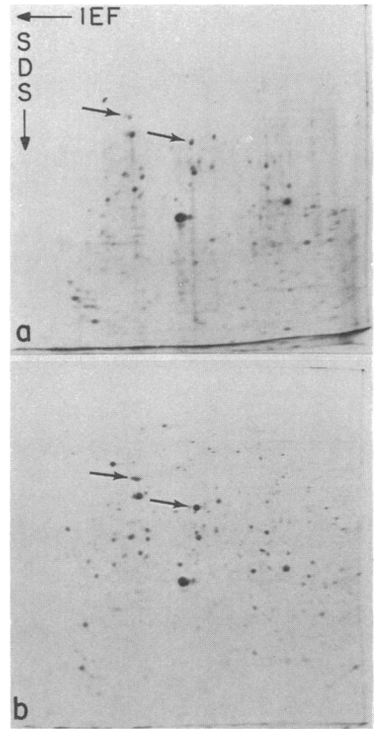

FIG. 7. Two-dimensional gel electrophoresis showing the induction of 89- and 68-kDa heat-shock proteins (arrows) in CHO cells as a result of release from anoxia. (*a*) Control pattern of protein synthesis; (*b*) pattern of protein synthesis 8 hr after reoxygenation (subsequent to a 24-hr anaerobic exposure). Equal masses of protein were loaded. Autoradiograms only are shown. IEF, isoelectric focusing; SDS, $NaDodSO_4$.

site of a longer exposure or of a release from a p76- and p97-induced state to obtain this result (unpublished data). Immediately after initiation of anaerobiosis, there is a slight decrease in total radiolabel incorporation into protein (cpm/mg of protein) followed by a nearly complete recovery under anoxia. However, if glucose is allowed to be exhausted during the anaerobic exposure, cell death rapidly ensues (as measured using colony survival) and cpm incorporation dramatically declines.

DISCUSSION

We have previously reported that the addition of glucose to glucose-deprived cells, which have induced levels of glucose-regulated proteins (p97 and p76), results in the induction of heat-shock proteins (10). We have further observed that the addition of glucose to glucose-deprived cells prior to the time of the induction of these glucose regulated proteins does not lead to the induction of heat-shock proteins (unpublished data). This appears to be analogous to the release from anoxia study presented here. It is of interest that in either case a brief period of deprivation is unable to induce p76 and p97, but rather lengthy exposures in these environments were required. The data presented here together with our earlier glucose studies collectively suggest that the glucose-regulated protein-induced state represents a sustained response to a deprived environment, while the heat-shock protein-induced state represents a temporary response, which results from a release from such an environment. An additional observation in the present study is the induction of the major heat-shock proteins as a result of the transition to anoxia (or as a result of a brief anaerobic exposure). However, the two studies differ in the following respect: in the work presented here, the anaerobic state is externally imposed while in our earlier study glucose deprivation was achieved by cell utilization. The fact that heat-shock induction is transitory in all of these instances (transition to and release from anoxia and release from glucose deprivation) suggests that the induced level of synthesis of these proteins functions only in adjusting the cell to a new environment.

The data presented in this study together with our earlier glucose deprivation work indicate that the major glucose-regulated and heat-shock proteins are under different controls, and one set is being turned off while the other is being turned on. This is significant, because we have previously recognized that heat shock itself can induce p76 (17, 18) in addition to the major heat-shock proteins; others have reported that proteins that appear identical to p76 and p97 are minor heat-shock proteins (16). It is possible that the control of the hyperthermic induction of p76 and p97 may be influenced by the environment of the cell at the time of heat shock. The separation of the induction of these proteins using deprivation and release experiments, as reported here, may present a more rigorous definition of these protein systems than does heat shock. Despite this distinction in the response of these two stress protein systems, the possibility still exists that the expression and/or repression of the major heat-shock proteins and p76 and p97 are in some manner coordinately controlled during environmental transitions.

Although the function of these proteins remains a mystery, a heat-shock protein of ≈70 kDa has been identified as the first major product of zygotic gene activity in mice (19). Furthermore, both of the protein systems discussed here appear to exhibit a significant degree of conservation (20, 21). The eliciting of particular stress protein systems as a function of change in environmental state may have important physiologic implications. The work reported here, together with our earlier study (10) showing the induction of p97 and p76 independently by either glucose or oxygen deprivation and the induction of heat-shock proteins upon release from either state, may contribute to the eventual understanding of the natural physiologic role of these protein systems.

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