Free Rad. Res. Comms., Vol. 9, No. 2, pp. 147–155
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EARLY ACTIVATION OF HEAT SHOCK GENES IN H_2O_2-TREATED DROSOPHILA CELLS

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(Received October 31, 1989; in revised form January 11, 1990)

Drosophila cells of a diploid clone derived from line Kc were treated with 1 mM H_2O_2 for 1 to 20 minutes. Dot blot and Northern blot analysis of RNAs extracted from control and treated cells showed that the transcriptional activation of the 6 heat-shock genes tested was early, and maximal within 5 minutes of H_2O_2 treatment. Analysis of the kinetics of induction of the heat-shock proteins (hsps) after an exposure to H_2O_2 of 2 or 5 minutes, followed by removal, suggests that this brief treatment was sufficient to trigger the synthesis of all the hsps, which was maximal 1.5 to 3h after this short H_2O_2 treatment.

KEY WORDS: Heat shock proteins, induction, reactive oxygen species.

INTRODUCTION

The heat-shock response to an elevation of temperature is a ubiquitous phenomenon, from bacteria to man.[1-8] It consists in the transcriptional activation of a set of specific genes, the heat shock genes, followed by the synthesis of the corresponding proteins, the heat shock proteins (hsps). Induction can be obtained by the addition of a wide variety of substances.[1,5] Thus, the heat-shock response can be considered as a cellular adaptation to environmental stress and deserves the general term of stress response. In these conditions, some controversy exists as to the nature and characterization of the intracellular factors which trigger the same response to very different chemical or physical stressing agents.[5-10]

Considering that the return to normoxia after a period of anoxia is sufficient for the rapid induction of hsps or stress proteins in Drosophila cells, we proposed that the plausible intracellular inducers were the reactive forms of the reduced species of oxygen (RFO) such as the superoxide ion (O_2^-) or hydrogen peroxide (H_2O_2).[11] Indeed among the numerous agents which are able to induce the hsps, many could act via changes of the redox potential of the cells.[1,5,12-14] One of the interests of our hypothesis is to tentatively establish a link between stress and the RFO-induced cellular damages which could in an ultimate phase lead to malignant transformation. The effect of H_2O_2 on mutation and cancer initiation has been reviewed.[15,16] We recently reported that a 3–5h treatment of Drosophila cells with H_2O_2[17] induces a decrease of global protein synthesis, an increase in the synthesis of actin and a slight

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increase in the synthesis of only two hsps (hsp70 and hsp23). We assume that under "physiological" conditions leading to an intracellular overproduction of oxy-radicals and H_2O_2, this RFO increase should be a very short-lived phenomenon since the cells are protected by an elaborate enzymatic defence system (e.g. via catalase). We therefore decided to study the effect of very short treatments of H_2O_2 on the transcription of hsp genes. We report here that all the hsp genes examined (hsp 84, 70, 27, 26, 23, 22 genes) are maximally activated after 5 min of H_2O_2 treatment whereas the corresponding proteins are maximally synthesised after 1.5 to 3 hours.

MATERIALS AND METHODS

Drosophila Cells

The diploid clone 23, derived from line Kc[18] was cultured with D22 medium supplemented with 5% fetal calf serum. The cells were grown in monolayers in Falcon plastic flasks at 23°C.

H_2O_2 Treatment

The cells were treated with 1 mM H_2O_2 at the beginning of the exponential growth phase. This final concentration was found optimal in a previous study.[17] After 1, 5, 10 or 20 min of treatment, the cells were prepared for RNA analysis. For protein analysis, the cells were rinsed in order to wash H_2O_2 off after 2 or 5 min of treatment and further incubated in conditioned medium.[17] The cells were thus allowed to recover for 1.5, 3 or 4.5h after this short H_2O_2 treatment, labelled with 1–3 MBq [^{35}S]methionine (Amersham, specific activity 50 TBq/mmol) during the last 90 min of recovery.

Protein Analysis

Labelled proteins were analysed on linear SDS-gradient gels of 6–14% acrylamide.[17] The autofluorograms were scanned with a Vernon photometric scanner. The peak corresponding to each hsp was determined after a study of kinetics of hsp induction by heat (not shown). The rate of synthesis of each hsp was calculated by measuring the area of the corresponding peak over the background.

RNA Extraction and Analysis

RNA was extracted by a procedure[19] devised for small quantities of cells. One flask (5×10^7 cells) was used for each experimental point. The culture medium over the monolayer of cells was discarded and replaced by 1 ml of lysis buffer (SDS 1% w/v, NP 40 1% w/v, EDTA 1 mM, dextran sulfate 50 μg/ml, in water). After 2 min of gentle agitation, the cells were detached with a rubber policeman. The total RNAs were phenol-extracted at room temperature, ethanol-precipitated and resuspended in water. For dot blot analysis,[20] extracts from control and H_2O_2-treated cells containing 1, 2, 4 μg total RNA were spotted on BA 85 nitrocellulose paper (Schleicher and Schüll). For the preparation of Northern blots, the RNAs from control cells and cells treated with 1 mM H_2O_2 for 5 min were separated by electrophoresis[17,21] and transferred to nitrocellulose paper. The dot blots and Northern blots were probed with

nick-translated plasmids. Six DNA probes were used, each containing one of the six heat-shock genes: hsp 22 (179 HB2), hsp 23 (179 1), hsp 26 (179 209), hsp 27 (179 55),[22] the hsp 70 Sal 1 fragment containing all the coding sequence, and the hsp 84 gene (from J. Lis, Cornell University, Ithaca, NY) (all probes are gifts of Prof. A. Tissières, Geneva).

RESULTS

Transcriptional Activation of the Hsp Genes

RNAs were extracted from control cells and from cells treated with 1 mM H_2O_2 for 1, 5, 10, 20 min. For a preliminary investigation, dot blots were probed with the genes hsp 84, 70, 27, 26, 23, 22. Under our experimental conditions, we did not detect any increase of hybridisation of the probes to RNAs from 1 min-treated cells as compared to the controls. Figure 1A shows that the hydridisation of the hsp probes was maximal

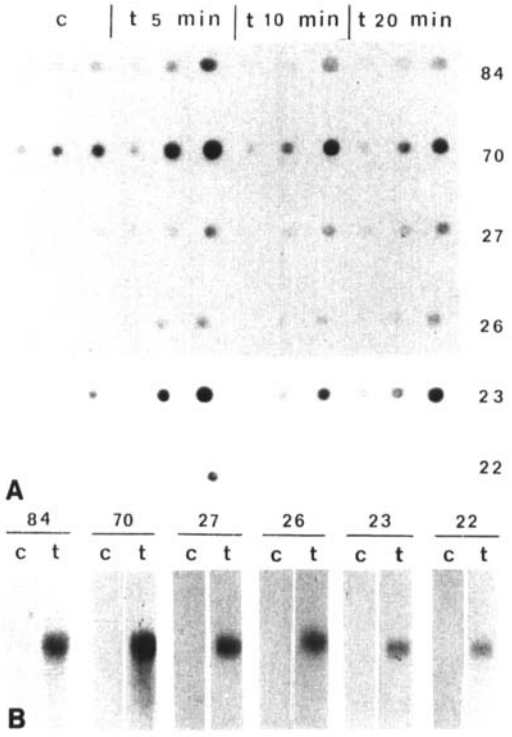

FIGURE 1 Dot blot (A) and Northern blot (B) analysis of RNAs from control cells (c) and cells treated with 1 mM H_2O_2 (t). The RNAs were extracted as described in Materials and Methods. A: H_2O_2 treatment was for 5, 10, 20 min. 1, 2, 4 μg total RNA were spotted on nitrocellulose and hybridised to nick-translated plasmids containing the hsp genes 84, 70, 27, 26, 23, 22. Autoradiographic exposure was for 24h (hsp84, 70, 27, 26) or 4 days (hsp 23, 22). B: H_2O_2 treatment was for 5 min. The RNAs were extracted, electrophoresed, transferred to nitrocellulose, hybridised, from left to right to the hsp probes 84, 70, 27, 26, 23, 22, and exposed for 20h. the portions of the different lanes showing hybridisation to the different probes were arbitrarily replaced on the same horizontal line for the commodity of presentation.

after 5 min of treatment. Therefore this time of treatment was chosen for a more precise analysis of the RNAs using the Northern blot technique. After electrophoresis and transfer of total RNA extracts from control, heat-shocked, and H_2O_2-treated cells, the nitrocellulose lanes were hybridised to the different probes (as described in [17]). The pattern of hybridisation was the same for RNAs from heat-shocked and H_2O_2-treated cells (not shown here, see [17]), each band migrating to the expected distance taking into account the known size of Drosophila hsp genes (see ref. [5], p. 19). For presentation, the bands were arbitrarily replaced on a horizontal line in Figure 1B. The hydridisation of all the probes tested to the RNAs from H_2O_2-treated cells can be seen. No hybridisation to the RNAs from control cells could be detected (except for hsp 84 with long exposures). This suggests an activation of transcription of the hsp genes in the H_2O_2-treated cells.

Synthesis of Hsp Proteins

When Drosophila cells were treated with 1 mM H_2O_2 for sevral hours,[17] a rapid and

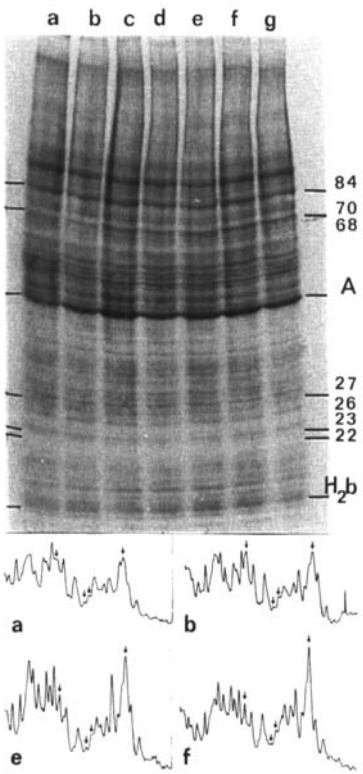

FIGURE 2 Protein synthesis in control cells (a) and cells treated with 1 mM H_2O_2 for 2 min (b–d) or 5 min (e–g). The cells were rinsed after the treatment, incubated in conditioned medium for 1.5h (b, e), 3h (c, f) or 4.5h (d, g) and labelled with [^{35}S]methionine during the last 90 min of incubation. The proteins were analysed by electrophoresis on 6–14% acrylamide gradient gels. Each lane was loaded with the equivalent of 9000 cpm acid-precipitable material. A, actin. The horizontal bars represent the hsps. Autofluorographic exposure was for 12 days. The densitometric scanning of the lower part of lanes a, b, e, f, is shown. The arrows indicate, from left to right, hsps27–26, hsp23, hsp22, H_2b.

substantial decrease of global protein synthesis, as measured by labelled-methionine incorporation, was observed. Following the observation that a 5 min-H_2O_2 treatment was sufficient to activate the transcription of all the hsp genes maximally, we examined the effects of short H_2O_2 treatments (2 and 5 min) followed by removal of H_2O_2 on the protein synthesis pattern of cells. Control untreated cells were rinsed and incubated in conditioned medium exactly as the treated cells (see Materials and Methods). Protein synthesis was followed at 1.5, 3, 4.5h after removal of H_2O_2. Under these conditions, we did not observe any change in the incorporation of labelled methionine following this short H_2O_2 treatment. Figure 2 shows the pattern of protein synthesis on SDS-PAGE gels. The induction of actin[17] is confirmed. A small increase in the synthesis of hsps 84, 70–68, 27–26 and histone H_2b can be observed in the cells treated with H_2O_2 for 2 and 5 min. Densitometric scanning and measurement of the areas under the peaks corresponding to hsps allowed more precise observations. At first, it must be recalled that besides hsp 84 and histone H_2b which are known to be relatively abundant at normal temperature,[23,24] the other hsps are synthesized at a low level in control cells (untreated cells, 23°C) (see Figure 2 and also [12,25–27]). Therefore, it was possible to evaluate the rate of increase of hsps in treated cells, taking as a reference the surface of the corresponding peaks in control cells. Table 1 shows the ratio for each hsp between the area of the peak over the background in treated cells and the one in the controls, under different stress situations. After a brief H_2O_2 treatment (2 min) followed by recovery of 1.5 to 3h, hsp84, hsp70–68 and hsps27–26 are increased approximatively 2 fold whereas hsps 23, 22, H_2b are not. After a 5 min H_2O_2 treatment followed by recovery, the synthesis of all the hsps is increased; hsp 70–68, 3 fold; hsps 84 and 27–26, 2 fold; hsps 22 and H_2b from 1.5 to 2 fold. The increase of hsp 23 is not significant and this hsp probably requires longer exposures to H_2O_2 as suggested earlier.[17] Although the increase of hsp synthesis in H_2O_2-treated cells is not as pronounced as that observed in cells exposed to an elevation of temperature (Table 1, right column), it is nevertheless significant.

DISCUSSION

The activation of one or several hsp genes by H_2O_2 and the induction of some hsps have been reported in several biological systems.[17,28–31] We now report the early increase of the transcripts of all the hsp genes studied (hsp 84, 70, 27, 26, 23, 22 genes) in Drosophila cells. This increase is maximal after 5 min of a 1 mM H_2O_2 treatment and probably begins earlier as suggested by the pattern of hsp synthesis in cells treated by H_2O_2 for only 2 min. the level of all the hsp transcripts then decreases after 10 min of H_2O_2 treatment. This very early and transitory reaction explains why, in a previous study,[17] we failed to see the increase of level of all the hsp mRNAs, after a 10 min or a 3h-H_2O_2 treatment. In fact, a short treatment of 2 or 5 min is sufficient to induce the transcripton of all the hsp genes with the accumulation of a small amount of hsp mRNAs which are later on translated. This translation appears as a small wave of synthesis of all the hsps (including histone H_2b).

The induction of the "heat shock" response that we observe after a H_2O_2 treatment has several points in common with that obtained by a heat treatment. Firstly, the kinetics of activation of the hsp genes by H_2O_2 are very similar to those observed after a heat shock.[32] Also, the increase in binding capacity of the heat shock transcription factor to the DNA heat shock regulatory element[33] (an essential step for the transcrip-

TABLE 1
Rate of increase of the different hsps in cells treated by H_2O_2 and in heat-shocked cells.

	H_2O_2 1 mM 2 min			H_2O_2 1 mM 5 min			Heat shock
		Recovery			Recovery		37°C
HSP	1h $\frac{1}{2}$	3h	4h $\frac{1}{2}$	1h $\frac{1}{2}$	3h	4h $\frac{1}{2}$	1h $\frac{1}{2}$
84	1.6 ± 0.1	2.0 ± 0.4	1.6 ± 0.2	1.6 ± 0.1	2.4 ± 0.2	1.4 ± 0.3	4 ± 1
70–68	1.5 ± 0.2	2.8 ± 0.5	2.4 ± 0.1	3.4 ± 0.3	3.2 ± 0.1	2.6 ± 0.3	22 ± 13
27–26	2.8 ± 0.8	2.5 ± 0.8	1.7 ± 0.4	2.2 ± 0.4	2.5 ± 0.7	1.5 ± 0.3	17 ± 10
23	0.9 ± 0.2	0.9 ± 0.3	1.0 ± 0.1	2.1 ± 0.8	1.5 ± 0.2	1.3 ± 0.3	18 ± 8
22	1.9 ± 0.7	2.1 ± 1.0	1.0 ± 0.1	2.0 ± 0.5	2.5 ± 0.1	1.4 ± 0.3	23 ± 11
H_2b	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.3	1.5 ± 0.1	2.2 ± 0.5	1.1 ± 0.3	6 ± 3

NOTE: The rate of increase of each hsp is expressed as ratio between the area of the peak over the background in treated cells and the one of the corresponding peak in the control cells. Values are means of separate experiments ± SD. $n = 3$.

tional activation of Drosophila hsp genes) begins within 30 sec of heat shock and reaches a plateau after 5 min.[34] Secondly, the kinetics of protein synthesis induced by H_2O_2 also resembles that observed following a heat shock. In Drosophila cells, the synthesis of hsps has been reported to being within 10 min of exposure to 37°C, while their maximal rate of synthesis was reached within 90–120 min and was followed by a decline.[25] Our experimental conditions did not allow us to detect the beginning of the synthesis, but the maximal rate was found to occur between 90 min and 3h and was also followed by a decline. Thirdly, we show that the individual hsps have different patterns of induction with respect to H_2O_2 requirement, level of transcripts, rate and level of protein synthesis. Such individual characteristics of induction of the different hsps in response to temperature elevation have already been reported.[35]

The main difference between hsp induction by H_2O_2 and induction by heat resides in the lower level of synthesis of the H_2O_2-induced hsps. This might be due to the transitory character of hsp mRNA induction. The decrease of hsp mRNA levels in the case of continuous exposure to H_2O_2 (20 min or more[17]) is an indication that H_2O_2, and possibly hydroxyl radicals resulting from a Fenton-type reaction[36] might be responsible for the loss and low level of the mRNAs. The inhibition by free radicals might also reside at some other step of the translation of the hsps. However, the translation of normal (other than hsp) mRNAs does not seem to be inhibited after a short H_2O_2 treatment as it is after a heat shock, as judged by the general pattern of protein synthesis (Figure 2). A lower level of hsp synthesis as compared to heat shock was also reported after anaerobiosis recovery in Drosophila Kc cells,[11,13] during recovery from ischemia in rat[37] or in cardiac myocytes after imposition of hemodynamic overload.[38] Thus, the responses to a heat shock or to an oxidative stress are not strictly parallel. The cellular response to a temperature elevation of several degrees (10 or 20°C) is spectacular[1-8] but it may represent a paroxysmal situation as compared to the different stress conditions encountered during the cellular life, where an oxidative stress might be more "physiological" than a heat shock. Whatsoever, even if an oxidative stress (here, a short H_2O_2 treatment) cannot support the large accumulation of hsps which is characteristic of a heat shock,[13] it is nevertheless sufficient to induce the stress response.

The nature of the stress-induced intracellular factors which trigger the onset of the "heat shock" or stress response, and the mechanism of this induction are still unknown. A role for dinucleoside polyphosphates considered as "alarmones" was first proposed a few years ago[9] but this hypothesis could not be confirmed.[10,17] It is now currently asserted that aberrant proteins resulting from stressing conditions are the inducers of the stress response (see for example [39,40]). If it appears undoubtful that aberrant proteins are involved in the stress response, some data make them unlikely candidates for its early triggering. For example, the activation of the heat shock transcription factor takes place after 30 seconds of a heat treatment and is independent of protein synthesis.[34] The results we report here emphasize the rapidity of the stress response. Moreover, they suggest an action of H_2O_2 at a very early site in the mechanism of induction of the hsp genes. Indeed, we recently found that H_2O_2 was able to induce the immediate activation of the heat shock transcription factor in cellular extracts *in vitro*.[41] Thus, these results are compatible with the hypothesis of the reactive forms of oxygen as inducers of the heat shock or stress response. We do not know if it is H_2O_2 itself or other free radicals which are concerned since H_2O_2 can be a source of free radicals, such as hydroxyl radicals derived from a Fenton-like reaction.[36] We do not know either the exact role of free radicals in the mechanism of activation of the heat shock transcription factor.

Here, it is worthwhile recalling that all the organisms that consume oxygen are exposed to the reactive byproducts of oxygen reduction which can cause many cellular damages[29,43] and which are indeed linked to several diseases and carcinogenesis.[15,16,42,43] We think we have designed a protocol which partially mimics what occurs during an oxidative stress where a sudden burst of oxy-radicals and H_2O_2 is immediately compensated by the enzymatic defence system of the cell. This experimental procedure allowed us to evidence the very beginning of a response equivalent to a heat-shock response, that is, the rapid triggering of transcription of the hsp genes and the beginning of synthesis of the hsps. This early activation of all the hsp genes seems sufficient to support the synthesis of all the hsps which becomes maximal 1.5 to 3h after the short exposure to H_2O_2.

The activation of heat-shock genes and synthesis of heat-shock proteins might thus be considered as one of the defence mechanisms against reduced species of oxygen-induced damage.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer and the Institut National pour la Santé et la Recherche Médicale (France).

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Accepted by Prof. H. Sies