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ON THE MECHANISM OF ACTION OF H_2O_2 IN THE CELLULAR STRESS

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We propose a hypothesis according to which the reactive and reduced species of oxygen could be the intracellular inducers of the stress (or "heat-shock") response. This hypothesis is based on the following observations on Drosphila cells: -a) the return to normoxia after 24 h anaerobiosis is sufficient to induce the synthesis of the "heat shock" proteins without elevation of temperature together with a rapid increase of O_2 consumption; -b) hydrogen peroxide introduced in the culture medium induces the early transcriptional activation of the "heat shock" genes (maximal after 5 minutes); -c) hydrogen peroxide added to cellular extracts in vitro (thus acting as an intracellular metabolite) activates instantaneously the binding capacity of a "heat shock" factor to a DNA "heat shock" regulatory element. Thus, hydrogen peroxide, and possibly other reactive reduced species of oxygen, could trigger the onset of the stress (or "heat shock") response.

KEY WORDS: Hydrogen peroxide, cellular stress, heat shock genes, transcription factors.

INTRODUCTION

The response of cells to a sudden increase of temperature implies a complex cascade of events including the activation of the transcription of a set of genes (heat shock genes) whose transcripts are translated into heat shock proteins (hsps).[1-4] Schematically this phenomenon is ubiquitous, from bacteria to man, and moreover can be mimicked, without any change of temperature, by adding to the environment of the cells different compounds which apparently do not share any obvious chemical property.[5,6] Thus, it seems reasonable to speak about cellular stress instead of heat shock, the latter being indeed a stress amongst many others. This more general definition coincides well with one of the plausible roles of the so-called "heat-shock proteins", indeed regarded as the protection of the cells precisely against the stresses which induced them. However, a very intriguing question remains: by which mechanism(s) the numerous and quite different stressing agents lead to the transcriptional activation of the same set of the so-called "heat shock genes". More precisely it is hoped to find how so numerous and different extacellular stresses can trigger a very limited number of intracellular molecular events leading to the activation of a few transcription factors (such as the "heat-shock factor" HSF) which binds to DNA regulatory sequences (e.g. the "heat-shock elements" HSE) allowing the transcription of the heat-shock genes (for reviews see [7,8]). In this paper we want to explain how we

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were led to propose that the molecular events which are involved in the very first steps of the pathways conducting from an extracellular environmental stress to the genes activation could be the intracellular reduced and reactive forms of oxygen.

MATERIALS AND METHODS

The diploid clones FC and 89 K derived from line *Kc*[9] were grown in monolayers in plastic flasks with 3 ml of D22 medium plus 5% fetal calf serum at 23°C. The thickness of the liquid was 1 mm. The gas phase was air (1 atm, 760 mm Hg). The maximal concentration of dissolved O_2 was calculated to be 250 μM (O_2 partial pressure: 150 mm Hg, Bensen coefficient 0.028). Oxygen consumption was measured with a Clark electrode. The rate of oxygen consumption was expressed as a function of O_2 concentration and the results allowed calculation of the kinetics parameters V_M and *Km*.[10] ^{35}S — labelled proteins were analysed by polyacrylamide gel electrophoresis.[11] For RNA analysis, dot blots and northern blots were hybridised to DNA probes containing each of the six heat shock genes 84, 70, 27, 26, 23, 22.[12] HSF activation was evidenced in cellular extracts[13] by specific binding to synthetic HSE oligonucleotides and analysed by gel retardation assay as described. [14]

RESULTS

The Kinetics Parameters of Oxygen Consumption of Drosophila Cells Cultured in Vitro

In a series of experiments,[10] using a Clark electrode, we measured the kinetics parameters of oxygen consumption of the cells. The maximal rate of oxygen consumption (V_M) was found to be equal to 0.2 $\pm$ 0.05 fmol. min^{-1} $cell^{-1}$ for a Km equal to 4 $\pm$ 1 μM. This last value shows that the cells are well protected against hypoxia. Indeed, knowing the concentration of oxygen dissolved in the growth medium (250 μM), it is clear that the O_2 partial pressure has to be diminished 250 μM: 4 μM = 62 fold to obtain a twofold decrease of the V_M.

The Shift from Anoxia to Normoxia is sufficient for the Induction of the Heat-Shock Proteins in Drosophila Cells

The values of the kinetics parameters of cellular oxygen consumption allowed us to design experiments which confronted the cells with a sudden increase of their O_2 uptake. Such was the case when the cells were cultured for 24 h in an anoxic state (nitrogen) and then put back in normal O_2 conditions (air). We found that following the return to normoxia -a) the V_M doubled while the *Km* remained constant, -b) the activities of superoxide dismutase and catalase did not increase, -c) the synthesis of the hsps occurred early although the culture temperature was kept constant (i.e. no "heat shock"). We therefore proposed[10] that sudden overprodution of the reduced and reactive forms of oxygen, transitorily outflanking the cellular enzymatic defences, might play a role in the chain of events leading to the activation of the "heat-shock" genes and to the induction of synthesis of the hsps.

Oxidative Stresses and Dinucleoside Polyphosphates

Lee *et al.*[15] studying the cellular stresses in Bacteria put forward two hypothesis. The first one was that most stresses, including heat shock, were oxidative stresses. The second hypothesis was the following: the dinucleoside polyphosphates (e.g. Ap_4A) could be the intracellular inducers of the hsps in prokaryotes. Agreeing for the reasons we mentioned above that the oxidative stresses were indeed of interest, we measured the variations of Ap_4A and other dinucleoside polyphosphates in stressed Drosophila cells. We did not succeed to establish a clear relationship between the concentrations of dinucleoside polyphosphates and the induction of hsps in these eukaryotic cells.[16] We therefore continued to search for an effect of the reactive forms of oxygen, which would not be mediated by the dinucleoside polyphosphates.

Hydrogen Peroxide Provokes the Early Activation of the Heat-Shock Genes

The addition of hydrogen peroxide to the growth medium resulted in the transcriptional activation of actin and only two hsp genes among the six expected and in the increase of synthesis of the corresponding proteins.[12] The first question which arose was whether this response was really due to H_2O_2 knowing that the cells have a non-negligible catalase activity[12,17]. Therefore H_2O_2 could act, as suggested[18], via the O_2 produced. We do not think this is the case for the following reasons. When the cells were put for 2 to 24 h in an atmosphere enriched in O_2, no change of the pattern of protein synthesis occurred. When catalase was introduced with H_2O_2, the immediate production of O_2 into the growth medium did not provoke any change in protein synthesis. Lastly, when H_2O_2 was added in association with aminotriazole, an inhibitor of catalase activity, the effect was greater than with H_2O_2 alone. Therefore we think that H_2O_2 and not O_2 is the inducer. However this does not explain why we observed the activation of only two hsp genes. Recently, we succeeded to answer this question. Instead of studying the variations of RNAs after long H_2O_2 treatments (up to 3 h), we designed Northern blot analysis after very short treatments. The results (in preparation) can be summarised as follows. After addition of H_2O_2 into the growth medium of the cells, we observed a transitory increase of the transcripts of all the heat-shock genes. This wave was maximal after 5 min of H_2O_2 treatment but tended to diminish if the treatment exceeded 10–20 min. We may thus conclude that, consistently with our first hypothesis, H_2O_2 could be one of the first and common triggers which initiates the cascade of events leading to the activation of the heat-shock genes.

However, since H_2O_2 was added to the cells externally it could be argued that it reacted with putative compounds in the growth medium or in the cellular membranes. In order to answer this question and moreover to demonstrate that H_2O_2 acts as an intracellular metabolite into the cells, we studied the effect of H_2O_2 added to cellular extracts *in vitro*.

Hydrogen Peroxide Provokes the Binding of the Heat-Shock Factor(s) HSF to the DNA Heat-Shock Regulatory Elements HSE.

In the promoter region of the heat-stock genes was found a consensus sequence which binds the so-called "heat-shock factor(s)" (HSF) and appeared to be a regulatory element, the "heat-shock element" (HSE).[19] Schematically, and at least in Drosophila, mouse and men, it is admitted that the preexisting inactive intracellar HSF is activated

by heat, then binds to the HSE, and that this protein-DNA complex plays a fundamental role in triggering transcription of the heat-shock genes.[7,8] The fact that the HSF is activated can be evidenced *in vitro* by its ability to bind short synthetic ^{32}P-labelled HSEs. The complex migrates more slowly than the free labelled HSE during electrophoresis in non-denaturing polyacrylamide gel retardation assays.[14] In a first series of experiments, we compared the HSF binding activity in control, heat-shocked and H_2O_2-treated cells. We did obtain a specific HSE-binding with extracts of heat-shocked cells and with those of H_2O_2-treated cells as well. The similarity of the relative retardation and affinity of the factors activated either by a heat-shock or by a H_2O_2 treatment led us to conclude that heat or H_2O_2 activate the same factor. Moreover, HSF activation by H_2O_2 was very rapid (less than 30 seconds). It should be noted that this early activation of the HSF by H_2O_2 is consistent with the early activation of transcription of the heat-shock genes obtained, as mentioned above, with the same compound. In another series experiments we prepared supernatants of crude cellular extracts, thus discarding growth medium and cellular membranes. Hydrogen peroxide, considered here as an intracellular metabolite, was added to the supernatants of control untreated cells whose HSF was inactive. In these conditions, we obtained a practically immediate activation of the HSF with a threshold concentration of 1 μM H_2O_2. This activation did not occur in the presence of added catalase.

All these data (in preparation) taken together are consistent and show that HSF activation by H_2O_2 is at least not mediated by molecules of the growth medium or of the cellular membranes. On the contrary, H_2O_2 reacts with some intracellular factor. It could be the HSF itself as suggested by the extreme rapidity of the phenomenon. Nevertheless, one cannot exclude the possibility that H_2O_2 reacts with an intracellular unknown factor which, in its turn, activates the HSF.

DISCUSSION

This rapid review of published or in preparation data of our laboratory had a goal: to illustrate our hypothesis that many of the quite different chemicals[5,6] which mimick the heat-shock (and likely the heat-shock itself[20,21]) during environmental stresses share the common property to disturb the redox state of stressed cells (see also[11,20-28]). One of the plausible pathways should involve as we noticed[10] the reduced and reactive forms of oxygen (RFO). In this sense these intracellular compounds may represent inducers of the stress (so-called "heat-shock") responses. More interesting is that these forms of oxygen could represent a link between cellular stresses and cellular pathologies that RFO can promote.[29,30] In the light of our work, that seems to be the case at least for H_2O_2.

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