H₂O₂-Induced Increases in Cellular F-Actin Occur without Increases in Actin Nucleation Activity[1]

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Previous work has shown that H_2O_2 causes an increase in polymerized actin (F-actin) inside cells. To test the hypothesis that increased polymerization resulted from a mechanism involving increased actin nucleation activity, we employed methods utilizing pyrene-labeled actin to quantify the actin nucleation activity of cell lysates and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)–phallacidin binding assays to quantify the amount of F-actin in P388D₁ cells. H_2O_2 increased polymerized actin (NBD–phallacidin assay) in a dose-dependent manner with an effective dose giving 50% response (ED_{50}) ≈ 1 mM. Five millimolar H_2O_2 caused a 1.6-fold increase in NBD–phallacidin staining. In contrast, actin nucleation activity decreased in a dose-dependent manner with a similar ED_{50}. Five millimolar H_2O_2 caused a 30–40% decrease in actin nucleation activity. The effect was rapid, occurring within 5 min of H_2O_2 addition. The results indicate that H_2O_2 causes cytoskeletal changes that enhance NBD–phallacidin binding without increasing actin nucleation activity. Fractionation studies showed that the nucleation activity in H_2O_2-treated cells and controls sedimented with the Triton X-100-insoluble cytoskeleton, and the cytosolic fraction appeared to contain an inhibitor of actin polymerization.   © 1994 Academic Press, Inc.

H_2O_2 generated from superoxide produced by activated leukocytes or produced endogenously by tissue cells may be an important initiator of cell death and tissue injury during inflammation. *In vitro*, H_2O_2 kills tissue culture cells (e.g., P388D₁ cells and endothelial cells) in conjunction with alterations of ATP levels, redox potential, and DNA strand breakage (1, 2). Recently, H_2O_2 also has been shown to initiate profound changes in the cell cytoskeleton which can be observed morphologically (3–7) and which correlate with an increase in polymerized actin (F-actin)[3] in the cells (3, 8). In addition, it has been shown that significant increases in polymerized actin occur primarily under conditions where sulfhydryl oxidation occurs in cells (8).

We are interested in understanding the mechanisms by which H_2O_2 induces this enhancement of actin polymerization. In models of cell migration (neutrophils and the slime mold, *Dictyostelium discoideum*), chemoattractant-induced increases in F-actin involve a mechanism which includes an increase in actin nucleation activity (9, 10). Actin nucleation activity (or actin nucleation sites) refer to the sites where monomeric actin is rapidly added to a growing polymer. We thus hypothesized that the increase in F-actin induced by H_2O_2 resulted from an increase in actin nucleation sites. One possible mechanism may be the triggering of signal transduction pathways similar to that seen in migratory cells (11), although in some cell systems oxidant stress appears to inhibit, not activate, signal transduction systems (12, 13). Alternatively, *in vitro* studies have shown that sulfhydryl oxidation of purified actin increases the rate of actin polymerization, possibly by enhancing the formation of nucleation sites (14). This suggests an alternative hypothesis, that H_2O_2 induces oxidation of sulfhydryls in actin, resulting in an increase in the formation of actin nucleation sites and thus increasing the amount of F-actin.

We show, on the contrary, that cellular increases in F-actin following H_2O_2 exposure correlate with a decrease

[3] Abbreviations used: NBD, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); ED_{50}, effective dose that gives 50% response; F-actin, polymerized (filamentous) actin; G-actin, monomeric (globular) actin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

in actin nucleation activity, suggesting that other mechanisms are responsible for the increases in actin polymerization.

METHODS

Preparation of cells. P388D$_1$ cells, a transformed murine macrophage cell line, were cultured utilizing standard culture conditions (4). These cells were chosen for these studies because of our extensive past experience characterizing the effects of H$_2$O$_2$ on their actin filament organization (3, 4, 8). The cells were harvested and resuspended at 2×10^6 cells/ml in modified Gey's buffer (4). Cells were incubated at 37°C, and H$_2$O$_2$ was added at various concentrations and/or for varying times from a stock solution of H$_2$O$_2$. The concentration of H$_2$O$_2$ in the stock was verified by measuring the optical density of the stock at 230 nm. At the end of the treatment time, 5000 U of catalase was added to consume any remaining H$_2$O$_2$, an aliquot was fixed for F-actin staining, and an aliquot was lysed for measurement of nucleation activity.

Measurement of F-actin content. Cells were fixed and stained by 1:1 dilution with a staining cocktail containing 7.4% formaldehyde, 0.2 mg/ml lysophosphatidylcholine as a permeabilizing agent, and 3.3 µM NBD–phallacidin which binds with high selectivity to F-actin, but not G-actin (monomeric actin) (15). The amount of bound NBD–phallacidin was quantified by flow cytometry (8, 16, 17).

Measurement of actin nucleation activity. Nucleation activity was measured as the ability of cell lysates to stimulate polymerization of pyrene-labeled G-actin (9). Actin was purified from rabbit muscle, then pyrene-labeled according to published procedures (18). Monomeric pyrene-actin was diluted to 2 µM into 250 µl of polymerization/lysis buffer (500 mM KCl, 1 mM MgCl$_2$, 5 mM DTT, 20 mM Hepes, 1 mM ATP, 1 mM EGTA, 0.2% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM PMSF, pH 7.4) followed immediately by addition of 50 µl of cells at 2×10^6 cells/ml to yield a final cell concentration of 3.3×10^5 cells/ml (unless otherwise indicated). As the pyrene-actin polymerizes, the fluorescence quantum yield of the pyrene moiety increases 20- to 30-fold (19). Fluorescence intensity was measured at 1- to 2-min intervals for 15 min. The excitation and emission wavelengths were 365 and 410 nm, respectively. The sample was illuminated only while taking a reading to minimize the possibility of photobleaching. All samples were allowed to polymerize completely (16–24 h) and the fluorescence intensity of the completely polymerized sample measured. Under the conditions of the experiments, the number of nucleation sites is proportional to the rate of polymerization and hence the rate of increase of fluorescence (9). The rate of polymerization was calculated as the slope of the fluorescence increase over 15 min normalized to the 100% polymerized value.

Localization of nucleation activity. Distribution of nucleation activity between the cytosol and the Triton X-100-insoluble cytoskeleton was determined by lysing cells in the polymerization/lysis buffer, then rapidly segregating the cytosol and Triton X-100-insoluble cytoskeleton by centrifugation at 10,000g for 30 s (9, 10). The supernatant (cytosol) was removed and pyrene-actin added to initiate the nucleation assay. Alternatively the supernatant was discarded and the pellet immediately resuspended in polymerization/lysis buffer, and pyrene actin added to initiate the assay. These samples were compared to samples that were lysed, centrifuged, and then remixed and pyrene actin added. The lysis buffer for these experiments contained 138 mM KCl rather than 500 mM KCl because the high KCl concentration interfered with segregation of the Triton X-100-insoluble cytoskeleton. There was a small decrease in nucleation activity of whole-cell lysates in 138 mM compared to 500 mM KCl lysis buffer. However, H$_2$O$_2$ dose–response curves were the same in either buffer.

To determine if DTT was reversing the effect of H$_2$O$_2$ in the samples that were centrifuged and remixed, DTT was removed from the lysis buffer. This had no effect on the H$_2$O$_2$-treated samples. Thus all data with or without DTT were pooled for the analysis of Table I.

Statistics. Data were compared using an unpaired two-tailed *t* test calculated with InStat software (GraphPad, San Diego, CA). A *P* value of 0.05 was considered statistically significant.

RESULTS

Pyrene-Actin Polymerization in the Presence or Absence of Cells

Figure 1 shows representative data of pyrene actin fluorescence at 37°C over a 15-min period in the presence or absence of cells. In the presence of cells the increase in intensity was linear over that period. In the absence of cells, little increase in intensity occurred. In general, the extent of spontaneous nucleation activity varied from one actin preparation to another, but was less than 20% that seen in the presence of control cells. Although minimal, this was subtracted from cell data to eliminate any contribution of spontaneous nucleation contributing to the calculation of the slope.

Cell Concentration Dependence of Nucleating Activity

The effect of cell concentration was determined as shown in Fig. 2. At low cell concentrations, the rate of increase in fluorescence intensity was proportional to cell concentration. However, above 3.3×10^5 cells/ml lysis buffer, the rate plateaued. This was not due to depletion of actin monomers in the assay since the increase of fluorescence with time was linear for all cell concentrations, even those above 3.3×10^5 cells/ml. In addition, the final polymerization value (after 24 h of polymerization) was always at least twice as high as the fluorescence at the
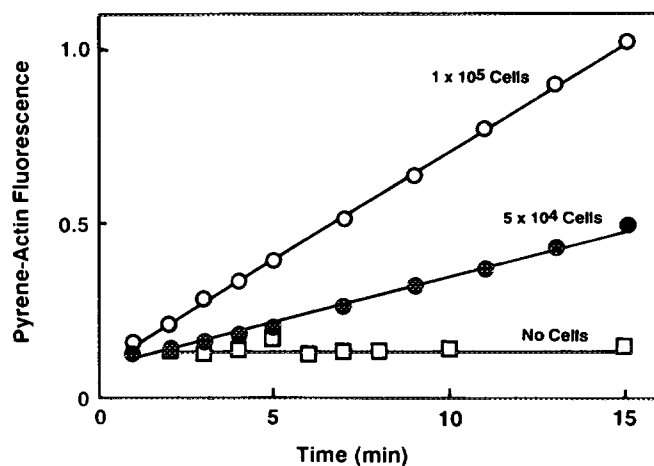
FIG. 1. Measurement of actin nucleation activity in P388D$_1$ cells. The fluorescence intensity of pyrene-labeled actin versus time in the presence or absence of cell lysates is shown. The total number of cell equivalents in the lysis buffer is indicated on the plot. The pyrene-labeled actin had little endogenous nucleation activity during the time course of the assay. The addition of cell lysates to the G-actin caused an immediate, linear increase in fluorescence intensity, indicating the presence of nucleation activity in the cell lysates.
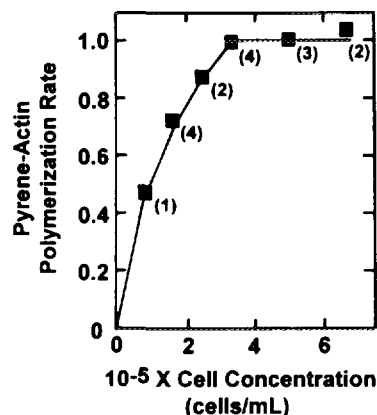
FIG. 2.   Cell concentration dependence of actin nucleation activity. Cells were diluted in standard buffer at cell concentrations between 0.5 and 4.0×10^6/ml. Fifty microliters of the cell suspension was then assayed, as described under Methods, to yield the cell concentrations in the lysis buffer as indicated in the x-axis. The rate of increase in fluorescence (slope of the fluorescence intensity vs time) was calculated and normalized to the fluorescence intensity of the completely polymerized sample. The data was further normalized so the rate of polymerization of the sample at 2×10^6 cells/ml was equal to 1. The values are the averages with the number of data points (different experiments) indicated in parentheses.

end of the 15-min assay. Thus the plateau seen at higher cell concentrations was presumably due to the presence of polymerization inhibitors which assume high concentrations over 3.3×10^5 cells/ml. At 3.3×10^5 cells/ml and below, this inhibitor appeared to be too dilute to influence the polymerization rate. This type of behavior has been seen when this assay has been performed with other cell types (9), although we have not attempted to further characterize this inhibitor in this study. For further studies, a final cell concentration of 3.3×10^5 cells/ml lysis buffer was utilized (a sample concentration of 2×10^6 cells/ml modified Gey's buffer) to optimize the ability to detect the nucleation activity but minimize the contribution of endogenous polymerization inhibitors.

Time Course of H₂O₂ Effects on Nucleation Activity

Nucleation activity was determined at various times after addition of 5 mM H₂O₂ to P388D₁ cells. To prevent possible interference of the assay by H₂O₂, catalase was added to the cells prior to beginning the nucleation assay. The nucleation activity dropped by ~40% within 5 min and remained depressed for 120 min (Fig. 3).

Concentration Dependence of H₂O₂ Effects on Actin Nucleation Activity and F-Actin Content

We determined the concentration dependence of H₂O₂ effects on nucleation activity and F-actin content after 5 min of treatment with H₂O₂. Actin polymerization was determined by flow cytometric methods and is shown in

Fig. 4A. The average amount of F-actin was determined as the mean channel number of the fluorescence histogram. Figure 4B shows the dose–response behavior of nucleating activity and F-actin. H₂O₂ caused an increase in F-actin content, but a decrease in nucleation activity. Thus, there was an inverse relationship between the two parameters. For the remainder of the experiments, 5 mM H₂O₂ was used as a concentration that gave maximal effect. This is also a dose of H₂O₂ that has been shown to produce significant sulfhydryl oxidation in these cells (8).

Distribution of Nucleation Activity between the Cytosol and Triton X-100-Insoluble Cytoskeleton

To determine the distribution of nucleation activity between the cytosol and Triton X-100-insoluble cytoskeleton, cells were lysed with Triton X-100, the insoluble cytoskeleton rapidly sedimented, and the activity in the supernatant and pellet assayed. When the supernatant and pellet were recombined and assayed, the net activity was lower than that measured for whole cells diluted into the lysis buffer. Thus the centrifugation procedure alone diminished the rate of polymerization of the components to ~68% of samples lysed but not sedimented. (Table I). Since the centrifugation procedure resulted in there being a ~40- to 50-s time interval between cell lysis and addition of pyrene actin (whereas the time delay was <2 s if cells were simply added to lysis buffer and pyrene actin added immediately to begin the assay) we suspected that time between cell lysis and addition of pyrene actin may be an important factor. We thus measured the nucleation rate for samples that were lysed, but the addition of pyrene-
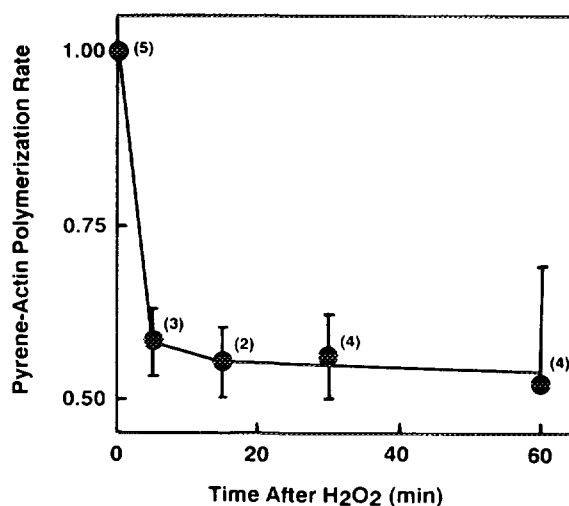
FIG. 3.   Time course of H₂O₂ effect on P388D₁ cell nucleation activity. Five millimolar H₂O₂ was added to P388D₁ cells for various times and nucleation activity quantified as described in Fig. 2 and under Methods. The nucleation activity was normalized to control cells not treated with H₂O₂. The numbers in parentheses indicate the number of experiments performed per data point. Error bars indicate ± standard deviation.
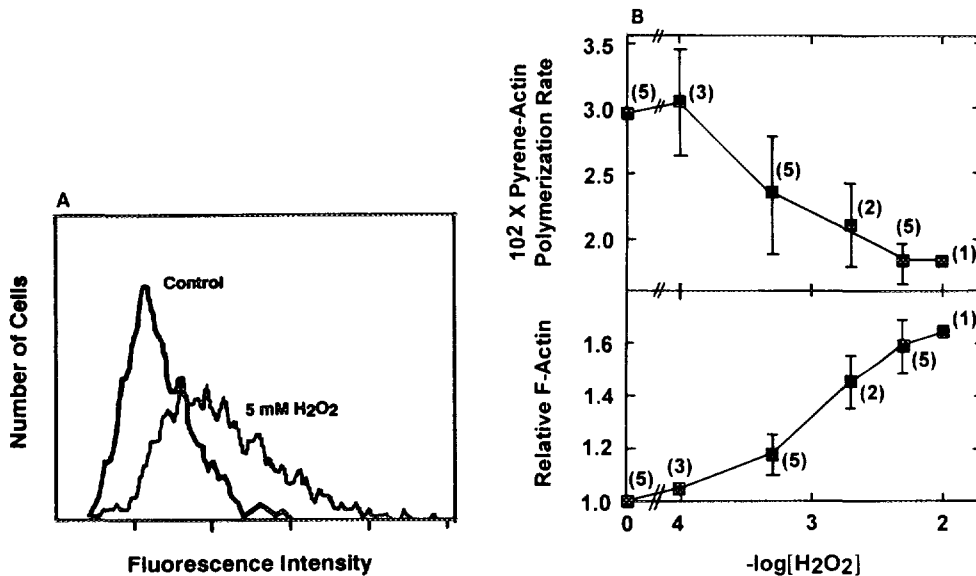
FIG. 4. H_2O_2 dose–response effects on NBD–phallacidin binding and actin nucleation activity. (A) P388D$_1$ cells were incubated in the presence or absence of 5 mM H_2O_2 for 5 min. Cells were fixed and stained with NBD–phallacidin and the fluorescence intensity quantified by flow cytometry, a method which measures the fluorescence intensity of individual cells. The data is presented as a histogram representing the number of cells (y-axis) that have a given fluorescence intensity (x-axis). Data from 1766 control cells and 1758 H_2O_2-treated cells are displayed. (B) Cells were treated for 5 min at the indicated H_2O_2 concentrations. NBD–phallacidin binding was quantified as the mean channel number of the flow cytometric fluorescence histograms and was normalized to the control (no H_2O_2). The nucleation activity is the polymerization rate expressed as the percentage of total actin polymerized/min. The numbers in parentheses by each data point are the number of times the experiment was performed and the error bars indicate the standard deviations. The concentration unit of the x-axis is molar.

actin delayed over a 2-min time course (Fig. 5). In this case, there was no centrifugation step. Nucleation activity decreased with greater time between lysis and addition of pyrene actin. The amount of time for the centrifugation step was $\sim$40–50 s. Thus the decrease in activity in centrifuged and recombined cytosol and pellets (compared to lysed but not centrifuged samples) could be explained by the time required to perform the centrifugation step. Thus approximately 50% of the nucleation activity of control cells appeared to be very labile, that activity being lost within a minute of cell lysis. If 10 min passed between the lysis of cells and the addition of pyrene actin, less than 15% of the nucleation activity remained in either control of H_2O_2-treated cells.

For cells treated with 5 mM H_2O_2, the nucleation activity of the lysed, sedimented, recombined samples was essentially the same as the cells lysed but not centrifuged (0.755 ± 0.215 vs 0.574 ± 0.365, respectively; Table I). The activity of the centrifuged and remixed samples from controls and H_2O_2-treated cells were the same (0.685 ± 0.582 vs 0.755 ± 0.215, respectively). This suggests that the pool of nucleation sites in controls that is labile to the time between lysis and addition of pyrene actin may be the same pool of nucleation sites that is lost upon H_2O_2 treatment (in the absence of cell lysis).

For either the control or H_2O_2 treated cells, segregation of the cytosol from the Triton X-100-insoluble cytoskeleton showed that the nucleation activity sedimented with the insoluble cytoskeleton (Table I; 1.732 ± 0.716 for control pellets and 1.303 ± 0.605 for H_2O_2-treated pellets), with no detectable nucleation activity in the cytosol (0.061 ± 0.301 for control cytosol and −0.035 ± 0.179 for H_2O_2 treated cytosol). Moreover, the activity measured in the pellet exceeded the activity measured in the recombined supernatant and pellet sample. For control cells, the sedimented and remixed cytosol and pellet sample (0.685 ± 0.582) was statistically different from the control pellet alone (1.723 ± 0.716) at a P value of 0.0011. For the H_2O_2-treated samples, the sedimented and remixed cytosol and pellet sample (0.755 ± 0.215) was statistically different from the H_2O_2-treated pellet (1.303 ± 0.605) at a P value of <0.01. This suggested that the cytosol may have contained a significant concentration of a polymerization inhibitor.

DISCUSSION

In P388D$_1$ cells, H_2O_2 induces the polymerization of actin when detected as an increase in NBD–phallacidin binding to F-actin, as a decrease in monomeric actin by the DNAse I assay, and as an increase in actin associated with the TX-100-insoluble extract detected by SDS–polyacrylamide gel electrophoresis (3, 8). Significant increases in F-actin occur primarily under conditions where sulfhydryl oxidation occurs in the cells (8). It has been postulated for *in vitro* systems that oxidation of Cysteine

373 on G-actin may stabilize nucleation sites and thus increase the polymerization rate of actin (14). Thus, we hypothesized that H_2O_2-induced actin polymerization in P388D₁ cells may result from an enhancement of nucleation sites.

Alternatively, in models of cell migration chemoattractant-induced increases in F-actin involve mechanisms which include an increase in actin nucleation activity (9, 10). Thus, it is possible that H_2O_2 could be inducing F-actin formation by stimulating existing cytoskeletal activation pathways involving enhanced nucleation activity similar to migratory cells. Either hypothesis, if operative in P388D₁ cells, would postulate a correlation between an increase in actin nucleation activity and increased F-actin. On the contrary, we found that NBD–phallacidin binding and nucleation activity were inversely correlated in H_2O_2-treated P388D₁ cells. This suggests that the pathologic mechanism of H_2O_2-induced actin polymerization is distinct from normal physiological processes. In addition, this lack of correlation between actin polymerization and actin nucleation activity invalidates the hypothesis that increased polymerization results from the creation of nucleation sites by direct sulfhydryl oxidation of actin in the P388D₁ cells.

In control cells, the nucleation activity was labile, in that the measured amount of nucleation activity decreased with increased time between cell lysis and addition of pyrene-actin, such that over 1 min ~50% of the nucleation activity was lost. This time-dependent decrease was not seen for H_2O_2-treated cells in that H_2O_2-treated cells
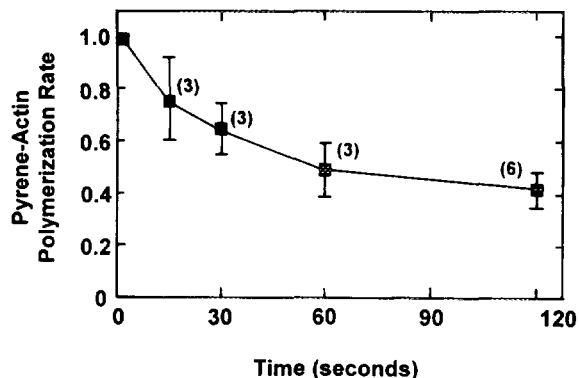

FIG. 5. Effect of time between cell lysis and addition of pyrene actin on the rate of pyrene actin polymerization. Cells were lysed into lysis buffer, and pyrene actin added at the indicated times after lysis. The pyrene actin polymerization rate was normalized to the standard protocol where the time between additions of cells and addition of pyrene actin was <2 s. The data points are the means of three separate experiments with error bars indicating the standard deviations.

that were lysed and assayed immediately gave the same nucleation rate as H_2O_2-treated cells lysed, centrifuged, and remixed prior to adding pyrene actin. This suggests that the pool of actin nucleation activity affected by H_2O_2 is equivalent to the labile pool in control cells.

If there is not an increase, and in fact a decrease of nucleation sites in the H_2O_2-treated cells, how can there be an increase in polymerized actin? The other major factor which could drive an increase in actin polymerization (even in the presence of a decrease in the number of nucleation sites) would be the release of G-actin from monomer-sequestering proteins such as profilin and thymosin β_4. It has been shown that dissociation of profilin from actin occurs by treatment with the sulfhydryl-reactive agent maleimide (20). This suggests that oxidation of sulfhydryls on actin promotes its release from profilin. That oxidized actin is still capable of polymerizing is evidenced by the fact that pyrene actin, which has an actin sulfhydryl attached to the pyrene moiety, is still capable of polymerization equivalent to controls (21). Thus we are pursuing our current hypothesis that oxidation of sulfhydryls on actin promotes its dissociation from profilin (and possibly thymosin β_4), and this increase in monomeric actin drives polymerization.

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TABLE I

Segregation of Nucleation Activity between Cytosol and Triton X-100-Insoluble Cytoskeleton

Conditions	Polymerization rate (normalized)[a]	
	No H_2O_2	5 mM H_2O_2
Cells lysed, not sedimented	1 (14)[b]	0.574 ± 0.365 (12)[b,c]
Cytosol + pellet, sedimented and remixed	0.685 ± 0.582 (10)[d]	0.755 ± 0.215 (8)[e]
Cytosol	0.061 ± 0.304 (14)	−0.035 ± 0.179 (13)
Pellet	1.723 ± 0.716 (14)[d]	1.303 ± 0.605 (13)[e]

[a] Numbers were normalized for each day's experiments to the condition of cells lysed, but not sedimented without H_2O_2 treatment. The mean and standard deviation for this sample without H_2O_2 was $1.11 \times 10^{-2} \pm 3.01 \times 10^{-3}$ %/min.

[b] Statistically different, $P = 0.0004$ based on the unnormalized data, utilizing an unpaired, two-tailed t test.

[c] Data are given as mean ± standard deviation with the number of experiments in parentheses.

[d] Statistically different, $P = 0.0011$, utilizing an unpaired, two-tailed t test.

[e] Statistically different, $P = <0.01$, utilizing an unpaired, two-tailed t test.

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