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A cellular model of oxidant-mediated neuronal injury

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Oxidants derived from the partial reduction of oxygen are thought to play a significant role in neuronal injury. We present here a cellular model of neuronal injury mediated by hydrogen peroxide (H_2O_2) using the PC 12 rat pheochromocytoma cell line. The organization of microtubules and microfilaments within neurites of PC 12 cells differentiated by exposure to nerve growth factor was examined after H_2O_2 injury using fluorescence microscopy. Concentrations of H_2O_2 as low as 100 μM produced an initial periodic pattern of microtubule depolymerization over 3-4 h which later progressed to complete depolymerization. Neuritic microspikes containing actin filaments were relatively more resistant to injury by H_2O_2 than microtubules. Blebbing of PC 12 cell bodies and neurites also was seen after H_2O_2 injury and the blebs appeared to contain microtubules. The destructive changes affecting neuritic structure preceded but were not essential for PC 12 cell lysis. Exposure of the cells to the Ca^{2+} ionophore, ionomycin (25 μM) also produced the same pattern of microtubule depolymerization in PC 12 neurites as was seen after H_2O_2 injury suggesting that H_2O_2 may mediate its destructive effect on the neurites via elevation of intracellular Ca^{2+}.

INTRODUCTION

Oxidants have been implicated in a variety of injuries to the central nervous system (CNS) including trauma and ischemia-reperfusion[2,6,8,11,19]. The generation of superoxide anion (O_2^-) during the reperfusion of ischemic brain has been demonstrated in cats by measuring the reduction of nitroblue tetrazolium[31]. Similarly, using the same technique, O_2^- production has also been demonstrated after fluid-percussion injury to the brain[20]. After the initiation of fluid-percussion injury to the CNS, progressive axonal damage has been noted, including degeneration, swelling and incomplete regeneration[28]. The mechanism responsible for the morphologic changes affecting neurites of the injured neurons is unknown.

A number of pathologic events affecting cellular morphology and the cytoskeleton have been demonstrated after oxidant exposure in non-neuronal cell types[9–11,13,17,23–25]. Exposure of hepatocytes to *t*-butyl-hydroperoxide leads to the Ca^{2+}-dependent formation of plasma membrane blebs[14]. Additional work has demonstrated that oxidants can induce oxidation of sulfhydryl groups in the microfilament protein actin[13,23–25], initiate actin assembly[11,13], and cause an ATP-dependent disruption of visible microfilament organization[9]. At concentrations of H_2O_2 which cause elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$), microtubule depolymerization has also been seen in injured endothelial cells[10].

To better define the effect of oxidant injury on the structure of neurites and the relationship of such pathologic changes to neuronal cytotoxicity, we have developed a model of oxidant-mediated neuronal injury using the PC 12 pheochromocytoma cell line which develops a neuronal phenotype after 5-7 days exposure to nerve growth factor (NGF)[5]. The advantages of this cellular model include the ability to perform studies of neuronal injury with a single cell type, and for studies of cytoskeletal pathology many features of the cytoskeleton in these cells have already been characterized (e.g. ref. 3 and 26). In this study we demonstrate that PC 12 cells differentiated to a neuronal phenotype by NGF are quite sensitive to injury by H_2O_2. Concen-

trations of H_2O_2 as low as 100 μM induce a characteristic pattern of tubulin depolymerization in PC 12 neurites which is accompanied by blebbing along affected neurites and cell bodies of the injured cells, and these cytoskeletal and morphologic changes precede cell lysis. In contrast, actin filaments within neuritic microspikes are relatively more resistant to visible damage by H_2O_2. The calcium ionophore, ionomycin, is capable of inducing identical changes in the microtubules of PC 12 neurites suggesting that H_2O_2-mediated changes in microtubule organization in PC 12 neurites are Ca^{2+} dependent. Finally, the rapidity with which low micromolar H_2O_2 induces lysis of NGF-differentiated PC 12 cells is in marked contrast to the observed kinetics of endothelial lysis[12,22], another potentially important target of oxidants in the CNS. This difference is not accounted for by a diminished capacity of differentiated PC 12 cells to metabolize H_2O_2 which is equivalent to that seen with endothelial cells.

MATERIALS AND METHODS

Description of model – culture methods

The PC 12 cell line (derived from a rat pheochromocytoma; American Type Culture Collection, Rockville, MD) has been used by many investigators as a cellular model of the neuron[3,5,26]. The undifferentiated PC 12 cells were cultured in either 75 or 150 cm^2 flasks pre-coated with rat tail collagen (0.01% in 0.1 M acetic acid, 6 μg/cm^2) and grown in RPMI 1640 media containing 5% fetal bovine serum (fbs) and 10% horse serum (hs) to which was added penicillin and streptomycin. They were passaged by shaking to dislodge adherent cells and cellular clumps were disrupted by vigorous pipetting prior to transfer.

For experiments with differentiated PC 12 cells, 6-well plates were initially plated at confluence with bovine pulmonary artery endothelial cells (AG2791A, National Institute of Aging – Aging Cell Culture Repository, Camden, NJ) for 48–72 h, after which the endothelial cells were removed by exposure to trypsin (0.05%) and EDTA (0.02%) for 15 min at 37°C. Any remaining endothelial cells were lysed by the addition of sterile deionized H_2O. We have found that the endothelial cells left a much better surface to support PC 12 adherence than did rat tail collagen and that this was equally important for the formation and spreading of neurites. After the pretreatment of the 6-well plates, the PC 12 cells were plated at approximately 1.75×10^5 per well in RPMI 1640 media containing 5% fbs and 10% hs for 24 h. The PC 12 cells were then allowed to differentiate for 5–7 days in RPMI 1640 containing 1% hs and 7S NGF (100 ng/ml; Sigma, St. Louis, MO) and were then ready for the injury studies.

Experiments in which the cells were exposed to a single bolus of H_2O_2 in 6-well plates were carried out using modified Gey's buffer (MGB) containing (in mM): NaCl 147, KCl 5, KH_2PO_4 1.9, Na_2HPO_4 1.1, $MgSO_4$ 0.3, $MgCl_2$ 1, HEPES 10, $CaCl_2$ 1.5 and glucose 5.5; at pH 7.4. In some experiments, the cells were exposed to a final concentration of 25 μM ionomycin for varying periods of time.

Fluorescence microscopy

Adherent cells were grown at subconfluence in 6-well plates (Falcon, Becton Dickinson). After varying periods of incubation at 37°C under the different experimental conditions, the adherent cells were fixed with 2% paraformaldehyde and placed on ice for 1 h. The paraformaldehyde was then removed and the cells were washed twice with MGB containing 0.1% bovine serum albumin (Sigma, St. Louis, MO). The cells were then incubated for 5 min with MGB containing 1% bovine serum albumin (BSA) at room temperature. After removal of the MGB plus BSA, the cells were permeabilized with 0.2% triton X-100 (Tx-100) for 5 min followed by another 5 min of exposure to MGB with 1% BSA after removal of the Tx-100. After an additional series of one to two quick wash steps with MGB plus 0.1% BSA, the cells were either stained with 165 nM rhodamine phalloidin (Molecular Probes, Junction City, OR) specific for F-actin in microfilaments, for 20 min at room temperature in the dark or exposed to the appropriate dilution of an antibody to tubulin within microtubules (1 μg/ml monoclonal mouse anti-β tubulin, Boehringer, Mannheim, Indianapolis, IN). After three quick rinses, a second rhodamine-conjugated goat antimouse IgG antibody (Boehringer Mannheim, Indianapolis, IN) 1:50 dilution, was then reacted with the samples to achieve visualization of the stained structures. Coverslips were sealed to each monolayer with 5 μl of 90% glycerol and the samples were viewed with an appropriate filter block on a Nikon optiphot fluorescence microscope. Fluorescence micrographs were taken at 400×magnification using TMAX-400 film (Kodak, Rochester, NY). Also, staining with a monoclonal antibody to acetylated α-tubulin (6–11B-1, a generous gift from Dr. Gianni Piperno, Mt. Sinai School of Medicine, New York, NY) at a 1:10 up to 1:50 dilution was done in a similar manner.

Scanning electron microscopy

Samples of whole fixed cells were prepared by fixation with modified Karnovsky's fixative (1.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) for 1–2 h at 4°C (fixative at room temperature was initially added to warm cells and later chilled to 4°C). Post-fixation with 1% OsO_4 was done in the same buffer for 1 h at room temperature. Adherent cells were dehydrated through graded ethanol, critical point dried and sputter coated with a carbon target. Samples were viewed with a scanning electron microscope at 20,000 acceleration voltage.

Quantitation of tubulin depolymerization within PC 12 neurites

The percentage of neurites demonstrating tubulin depolymerization from a given experimental sample (one well of a 6-well plate) was determined by counting 100 individual neurites (stained with anti-β-tubulin) at 400×magnification. Only neurites showing the characteristic pattern of tubulin depolymerization depicted in Fig. 2 of the Results section were counted as showing tubulin depolymerization in the determination. Also, care was taken to trace the course of individual neuritic processes so that the same process would not be counted more than once.

Viability determinations

The Live–Dead assay (Molecular Probes, Inc., Junction City, OR) was used to determine viability because it reduces manipulation of the adherent, differentiated PC 12 cells. The assay utilized two different fluorophores, ethidium homodimer and calcein-AM, both of which could be easily visualized with a fluorescent microscope using a fluorescein-compatible filter block. Ethidium homodimer, a DNA stain, stained the nuclei of dead cells a bright red color. Calcein-AM, the acetoxymethyl ester of calcein was taken up and cleaved by non-specific esterases to the fluorescent (green color)-free acid form only by viable cells. Utilizing 8 μM ethidium homodimer and 4 μM calcein-AM we were able to achieve good discrimination between dead and alive cells after a 15-min incubation at 37°C. One hundred adherent cells per well in situ were then counted, and the percentage of viable (green) cells was determined. Controls in which adherent PC 12 cells underwent detergent lysis with Triton X-100 demonstrated 100% ethidium staining and consistent loss of the ability to concentrate and cleave calcein-AM.

H_2O_2 consumption measurements

To compare the overall status of cellular antioxidant defenses in adherent, differentiated PC 12 cells with adherent endothelial cells, the rate of H_2O_2 consumption was determined by the technique of Hyslop and Sklar[15]. Briefly, the non-fluorescent substrate p-hydroxyphenylacetate (PHPA, Sigma Chemical Co., St. Louis, MO) was oxidized to the stable fluorescent product 2,2′-dihydroxybiphenyl-

5,5'-diacetate, (PHPA)$_2$, by the enzymatic reduction of H$_2$O$_2$ by horseradish peroxidase (Type IV Sigma). After adherent cells were exposed to a range of H$_2$O$_2$ concentrations, 10–50 μl of supernatant from each well (6-well plates) was added to 180.5 μg/ml PHPA and 58 μg/ml of horseradish peroxidase in 2 ml of modified Gey's buffer. Assays were performed in plastic cuvettes containing a magnetic starhead stir bar. The samples were placed in a stirred thermostatted sample chamber of an SLM series 8000C spectrofluorometer at 37°C. Excitation and emission wave lengths were 323 and 400 nm, respectively.

RESULTS

H$_2$O$_2$ induces depolymerization of microtubules in PC 12 neurites

PC 12 cells which had undergone differentiation in the presence of NGF for 5–6 days demonstrated a uniform pattern of microtubule staining within their neurites using a monoclonal antibody to β-tubulin (Fig. 1). When PC 12 cells were exposed to a bolus of H$_2$O$_2$ (final concentration ranging from 100 μM to 5 mM), characteristic changes affecting microtubule organization developed over a 3–4 h time course. Initially, the homogeneous microtubule staining with the antibody was lost and was replaced by a pattern of discontinuous staining which demonstrated a high degree of periodicity (Fig. 2). Later in the course of injury, the remaining areas of intense microtubule staining were also lost leaving a 'ghost-like' neurite remnant (Fig. 3). This pattern of events affecting tubulin was consistent with tubulin depolymerization which was initiated at different points along the long axis of the neurite. Blebbing of the PC 12 cell bodies and along neurites also oc-

curred after exposure to H$_2$O$_2$. The blebs appeared to contain tubulin (Fig. 4). Scanning electron microscopy demonstrated fragmentation of neurites in injured PC 12 cells at time points when depolymerization of neuritic microtubules was nearly complete (Fig. 5).

When the percentage of neurites (from 100 neurites counted per well) which showed some degree of disruption (tubulin depolymerization) were quantitated, an interesting injury pattern became evident. The entire dose range of H$_2$O$_2$ studied was capable of disrupting most of the neurites by 4–6 h. Higher concentrations of the oxidant produced the changes more rapidly, although the net effect did not appear to be different from that produced by the lower concentrations over a longer time course (Fig. 6).

Microfilaments and H$_2$O$_2$ injury to PC 12 cells

Since microfilaments are known to be early, prominent targets of oxidant injury in other cellular models[9–11,13,23–25], we also examined microfilament organization in PC 12 cells exposed to H$_2$O$_2$. Uninjured PC 12 cells stained with rhodamine phalloidin (which binds to polymerized actin) demonstrated prominent microspikes containing polymerized actin on some cell bodies and also along neurites which were oriented perpendicular to the long axis of the neurite (Fig. 7A). H$_2$O$_2$ exposure damaged many of the neuritic microspikes, although some microspikes remained relatively unchanged after injury even at time points when microtubule depolymerization was largely complete

Fig. 1. Fluorescence micrograph of uninjured (control) PC 12 cells stained using a double antibody technique for tubulin (murine monoclonal antibody to β-tubulin followed by rhodamine-conjugated goat anti-mouse IgG). 300 × magnification.
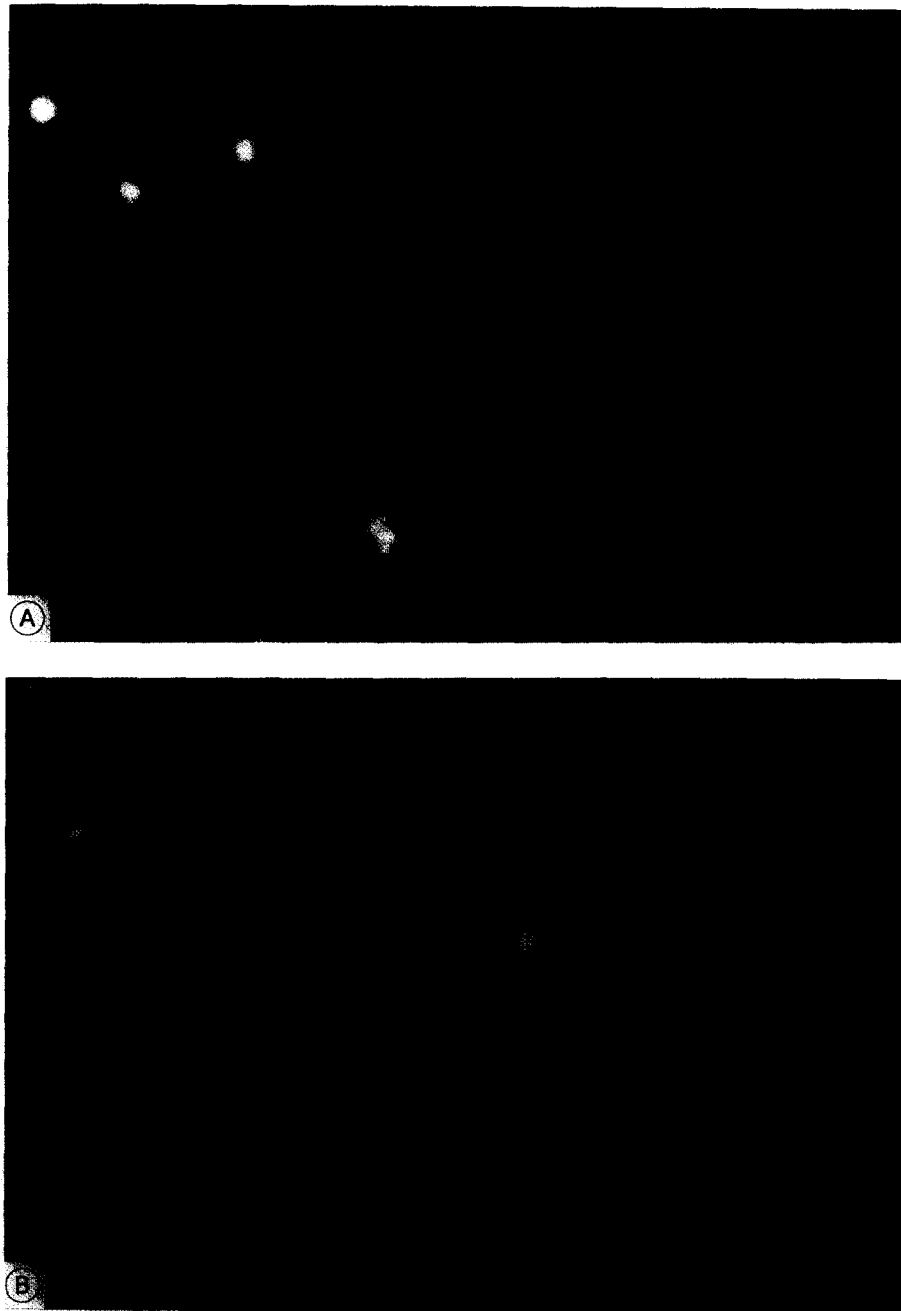
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Fig. 2. Fluorescence micrographs of PC 12 cells after H_2O_2 exposure stained using a double antibody technique for tubulin. A: microtubule organization in neurites 3 h after exposure to 250 μM H_2O_2. Note the periodic breaks in microtubule staining along the injured neurites. 300 × magnification. B: microtubule organization in neurites 4 h after exposure to 100 μM H_2O_2. Note the periodic breaks in microtubule staining along the injured neurites. 300 × magnification.

(Fig. 7B). In fact, microspikes containing F-actin often appeared to be the only remaining elements in many of the injured neurites at later time points.

Hypothesis: microtubule depolymerization within H_2O_2-injured neurites is initiated by elevation of cytosolic Ca^{2+}

Earlier work with P388D₁ and endothelial cells demonstrated that H_2O_2 can cause elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) levels[10,14]. Microtubule depolymerization occurred in adherent endothelial cells under

conditions (exposure to either $H_2O_2 > 5$ mM, or to the Ca^{2+} ionophore, 5 μM ionomycin in the presence of metabolic inhibition) which produced significant increases in $[Ca^{2+}]_i$ in the range of 1 μM or greater as measured with the fluorescent Ca^{2+} indicator, Quin 2[10].

We hypothesized that if elevation of $[Ca^{2+}]_i$ was responsible for the dramatic changes in microtubule organization within neurites of PC 12 cells exposed to H_2O_2, elevation of $[Ca^{2+}]_i$ independent of oxidant
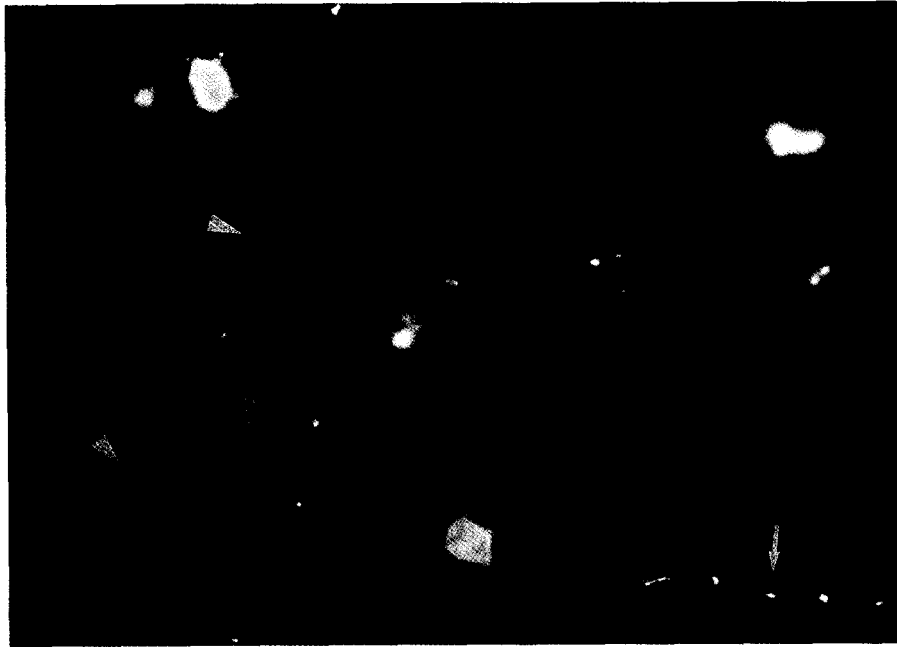
Fig. 3. Fluorescence micrograph of PC 12 cells after H_2O_2 exposure stained using a double antibody technique for tubulin. PC 12 cells 4 h after addition of 250 μM H_2O_2. Note the loss of tubulin staining in the neurite remnant (arrowhead) as compared to the periodic pattern of tubulin staining present on another neurite nearby (arrow). 300×magnification.

exposure should reproduce the same pathologic changes. Adherent PC 12 cells exposed to 25 μM ionomycin for 5–30 min demonstrated rapid disruption of microtubules within almost all (90%) neurites after only 15 min of exposure to the Ca^{2+} ionophore (Fig. 8A). The depolymerization of microtubules in PC 12 neurites induced by Ca^{2+} ionophore also exhibited the same high degree of periodicity as that seen following

H_2O_2 injury. When PC 12 cells were exposed to 25 μM ionomycin for 15 min in MGB prepared without the addition of Ca^{2+} plus the addition of 100 μM EGTA to further deplete Ca^{2+}, depolymerization of tubulin within neurite microtubules did not occur (Fig. 8B). The Ca^{2+}-depleted conditions alone did not affect PC 12 morphology or the integrity of neuritic microtubules (Fig. 8C).

Fig. 4. Fluorescence micrograph of PC 12 cells after H_2O_2 exposure stained using a double antibody technique for tubulin. PC 12 cells 4 h after addition of 500 μM H_2O_2. Note the brightly stained blebs on cell bodies and neurites (arrows). 300×magnification.

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Viability of PC 12 cells after H₂O₂ injury

We next examined changes in the viability of PC 12 cells after H_2O_2 exposure to determine the relationship between neurite disruption and neuronal death after oxidant exposure. To minimize manipulation of the cells we used a relatively new fluorescent method (Live–Dead Assay, Molecular Probes, Inc., Junction City, OR; see Materials and Methods). Experiments with exposure to 0–5 mM H_2O_2 over a 6-h time course revealed that there was a dose-dependent effect on the rate of PC 12 cell killing. Doses of H_2O_2 as low as 100 μM killed nearly 40% of the cells by 6 h (see Fig. 9).

Comparison of the data in Fig. 9 with that in Fig. 6 above demonstrates that in general, neurite disruption
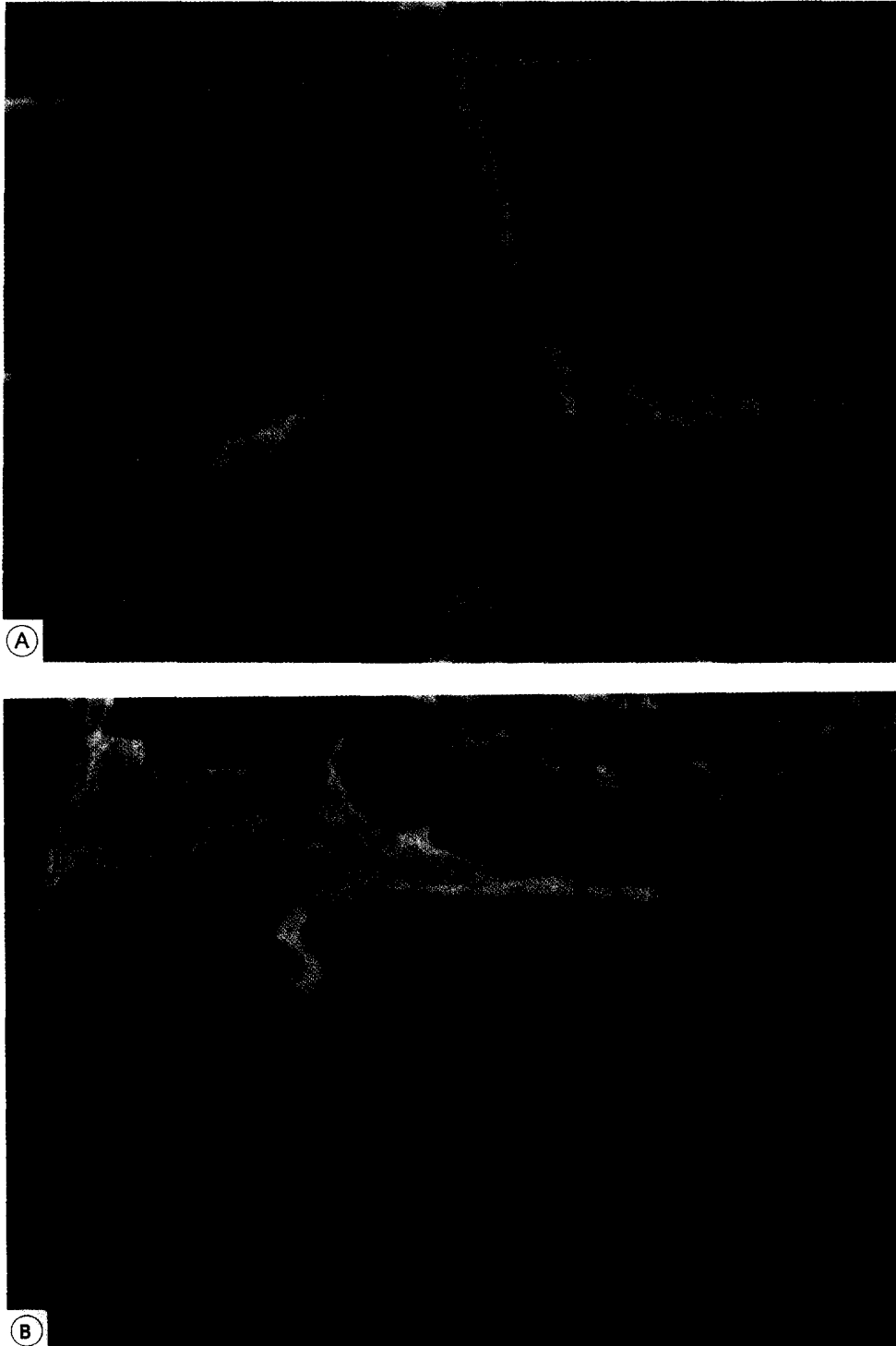

Fig. 5. Scanning electron micrographs of PC 12 cells. A: uninjured controls. 3500×magnification. B: 3 h after addition of 100 μM H_2O_2. 3500×magnification. Note the fragmentation and apparent flattening of the injured neurites.

preceded loss of PC 12 cell viability and was consistent with the hypothesis that neurite loss or disruption after injury may play an important role in neuronal death. To test this hypothesis, we examined the effect of the microtubule-stabilizing agent, Taxol[29] on neuritic morphology and PC 12 cell viability over a 6-h time course of oxidant injury. Exposure of PC 12 cells to 5 μM Taxol (added 15 min before oxidant addition) during the time course of H_2O_2 exposure prevented the microtubule depolymerization seen with H_2O_2 injury (Fig. 10A–C). Stabilization of microtubule morphology with Taxol did not alter the rate or extent of killing of PC 12 cells by H_2O_2 (Fig. 11). Thus, neuritic microtubule depolymerization is not an essential intermediate step in PC 12 cell lysis induced by H_2O_2.

Antioxidant activity of PC 12 cells

The rapidity with which low micromolar concentrations of H_2O_2 (e.g. 100 μM) produced cytotoxicity in the differentiated PC 12 cells was in striking contrast to the kinetics of H_2O_2-mediated killing of endothelial cells by similar concentrations of the oxidant[10,19]. Endothelial cytotoxicity secondary to low micromolar concentrations of H_2O_2 was typically manifest by 18–24 h rather than 4–6 h as was seen in the PC 12 cells. This observation led us to question whether the two cell types had different abilities to consume exogenous H_2O_2.

Adherent, differentiated PC 12 cells grown in 6-well plates as described above were compared with equivalent numbers of adherent endothelial cells (6.6 ± 0.4 × 10^5/well vs. 6.4 ± 0.1 × 10^5/well, respectively; $n = 5$)

with respect to their ability to consume a given load of H_2O_2 (100 μM and 1 mM) given as a bolus. Disappearance of the added H_2O_2 from the cellular buffer was monitored fluorometrically using the assay of Hyslop and Sklar[15] (Fig. 12A,B). The measurements were also conducted in the presence of 12 mM 3-amino-1,2,4-triazole[30], the potent inhibitor of catalase, to determine catalase-dependent H_2O_2 consumption.

There were no obvious differences in the pattern of H_2O_2 metabolism by either cell type even with relatively low (100 μM) or high (1 mM) concentrations of H_2O_2. Catalase appeared to be responsible for almost all H_2O_2 consumption by both cell types under the adherent conditions and at both concentrations of the oxidant.

DISCUSSION

In this study we have demonstrated that PC 12 cells differentiated by treatment with NGF to a neuronal phenotype are very sensitive to injury by H_2O_2. Microtubule depolymerization in the majority of PC 12 neurites was initiated by exposure to concentrations of H_2O_2 as low as 100 μM. Higher concentrations of H_2O_2 were associated with a more rapid progression and development of the neuritic disruption but the net effect of the lower concentrations appeared to be the same as the higher. Depolymerization of neuritic microtubules and plasma membrane blebbing along the neurites and cell bodies of injured cells preceded cell lysis. Experiments with the microtubule-stabilizing agent, Taxol, demonstrated that microtubule disrup-
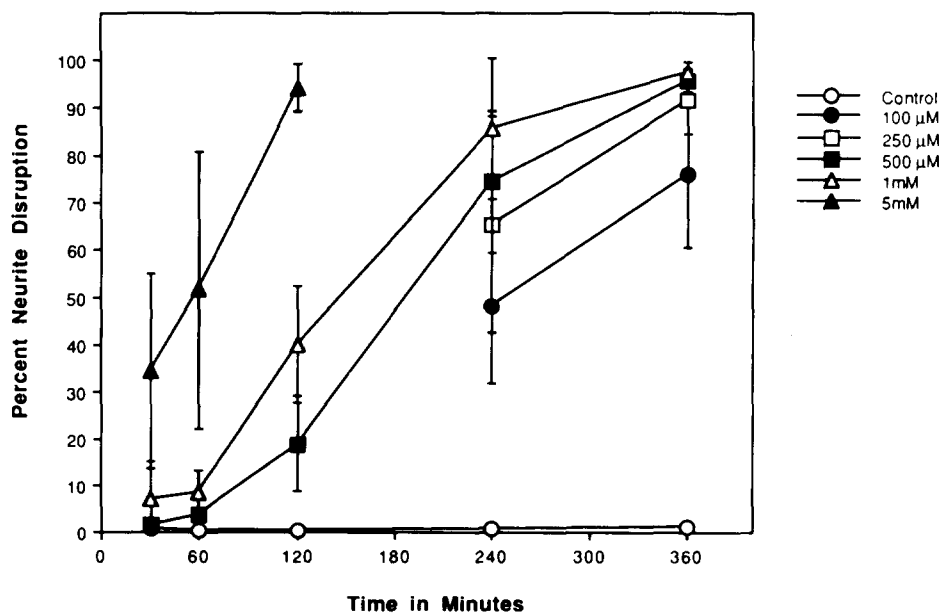

Fig. 6. PC 12 neurite disruption (microtubule depolymerization) after H_2O_2 exposure. Each data point represents the mean ± S.D. of $n = 5$ separate determinations.
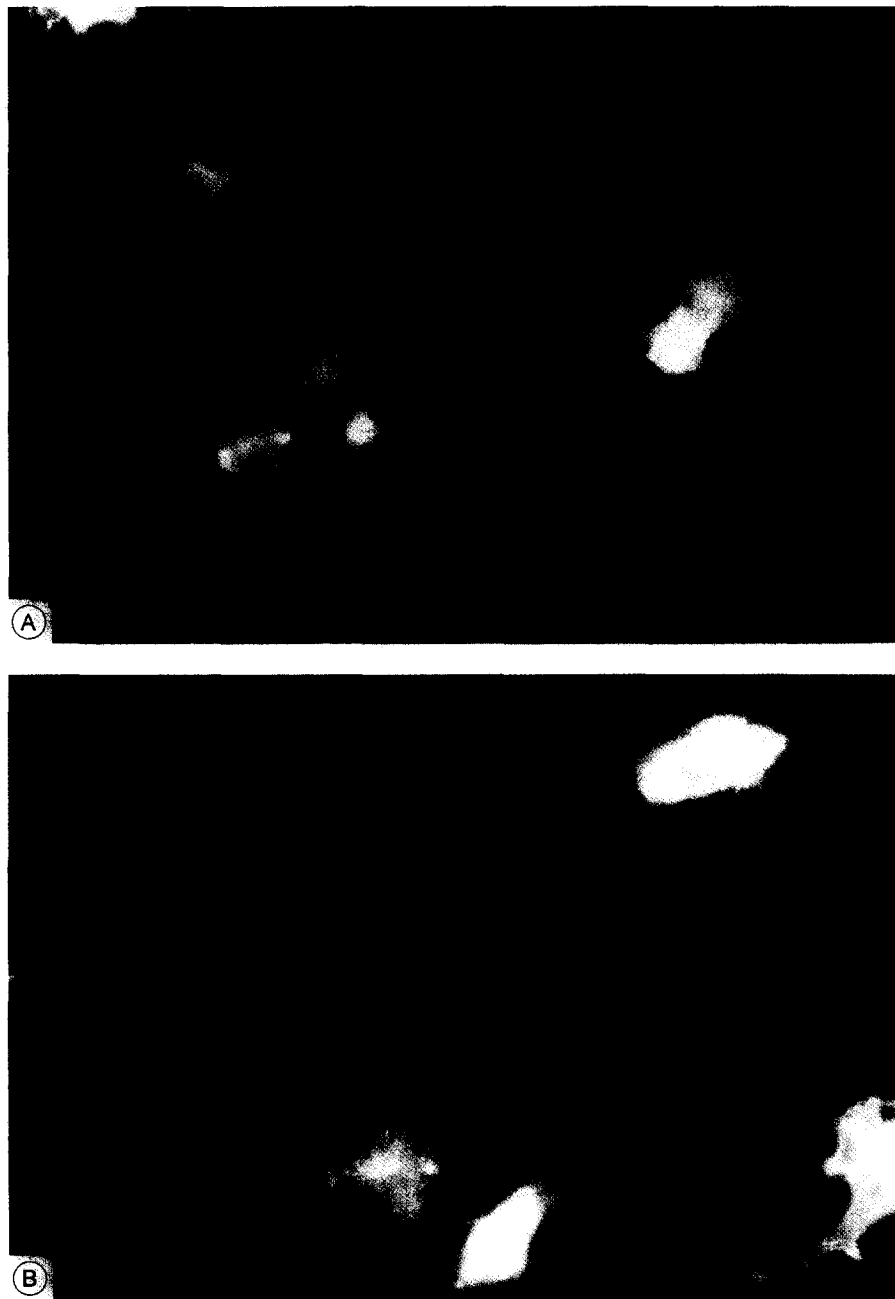
Fig. 7. Fluorescence micrographs of PC 12 cells stained with rhodamine phalloidin. A: uninjured controls. Note the prominent microspikes (arrows) which contain polymerized actin. 300×magnification. B: PC 12 cells 4 h after addition of 500 μM H_2O_2 stained with rhodamine phalloidin. Note the persistence of some microspikes where essentially all other components of the injured neurite have disappeared (arrow). 300×magnification.

tion within injured neurites does not play a necessary role in PC 12 cell lysis induced by H_2O_2.

It is interesting that there was an apparent flattening as well as fragmentation of neurites from injured

PC 12 cells (seen in scanning electron microscopy) at time points when oxidant-induced microtubule depolymerization was nearly complete. This implies that the normal three-dimensional architecture of the neurite

Fig. 8. Fluorescence micrographs of PC 12 cells stained with a double antibody technique for tubulin. A: the cells were exposed to Ca^{2+} ionophore (25 μM ionomycin) for 15 min prior to fixation and staining. Note the almost universal disruption (depolymerization) of microtubules in neurites and its similarity to the pattern seen with H_2O_2 injury. B: the cells were exposed to identical conditions as in A except the experimental buffer did not contain added Ca^{2+} and was further depleted of Ca^{2+} by the addition of a final concentration of 100 μM EGTA at pH 7.4. C: the cells were exposed to the altered buffer conditions in B without exposure to ionomycin. 270×magnification.

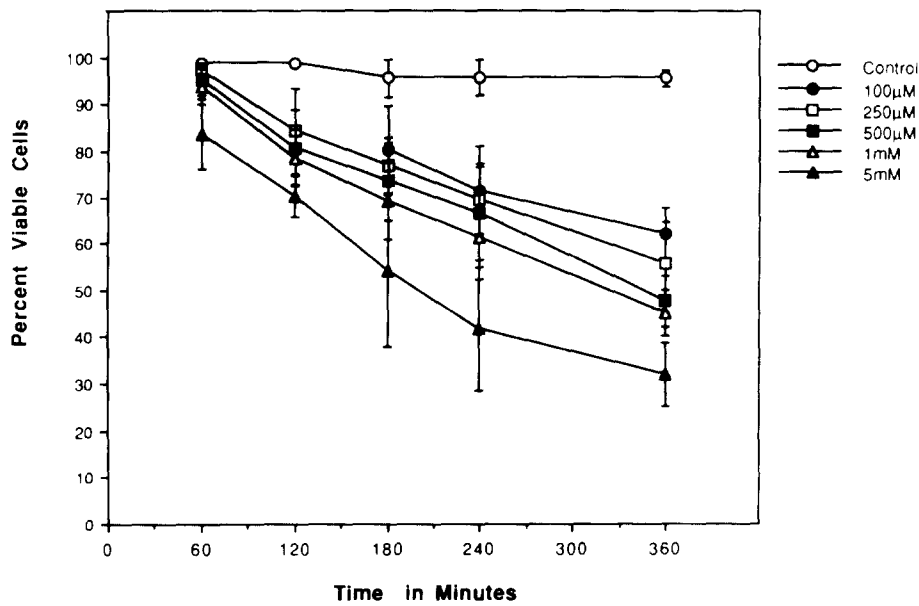Fig. 9. Effect of H_2O_2 on PC 12 cell viability. Each data point represents the mean ± S.D. of $n = 5$ separate determinations.

largely depends upon the presence of intact micro-tubules. The relative stability of actin filaments present in neuritic microspikes in response to H_2O_2 was in contrast to the sensitivity of actin filaments in other non-neuronal cell types to oxidant-mediated disruption[9-11,13,23-25]. Visualization of the actin-containing cytoskeleton in the cell bodies of PC 12 cells was limited in these experiments. It appears that actin filaments in microspikes may represent actin-containing structures which are relatively more resistant to oxidant stress.

The pattern of tubulin depolymerization induced by H_2O_2 injury in the neurites of differentiated PC 12 cells is quite interesting. The Ca^{2+} ionophore, iono-mycin, was capable of inducing Ca^{2+}-dependent micro-tubule depolymerization in neurites of treated cells in a manner similar to H_2O_2, although the kinetics of the process were much faster than for the oxidant. This supported the hypothesis that H_2O_2 disrupts $[Ca^{2+}]_i$ homeostasis in differentiated PC 12 cells and that the oxidant-mediated process occurs more slowly and with less uniformity than that seen with ionomycin treatment. These observations with PC 12 cells are not inconsistent with our earlier findings in the $P388D_1$ cell line demonstrating that oxidant stress can cause a release of Ca^{2+} from an intracellular pool, thus alter-

ing the set point for Ca^{2+} within injured cells[14]. Further confirmation of this hypothesis will require direct monitoring of $[Ca^{2+}]_i$ in PC 12 neurites during H_2O_2 injury. Colchicine, 5 $\mu g/ml$ (data not shown), induced a similar pattern of microtubule disruption after 2 h in neurites to that seen with ionomycin (Fig. 8A) or after H_2O_2 exposure (Fig. 2B) and progressed after 4-6 h to nearly complete loss of immunostaining for tubulin which also occurred as injury progressed after exposure to ionomycin or H_2O_2. This observation provided further confirmation that the injury was inducing depoly-merization of neuritic microtubules. The highly ordered pattern of depolymerization seen with ionomycin or H_2O_2 in these experiments implies that the organization of tubulin within individual microtubules somehow favors a highly periodic distribution of initiation sites for depolymerization. Recent work demonstrating the presence of tubulins within neurites which have been altered by post-translational modification to a more stable form (e.g. acetylated a-tubulin) suggests that there may be sites within a given tubulin polymer which are more or less stable to the effects of cold or elevated $[Ca^{2+}]_i$[3]. Antibodies directed at some of these modified tubulins have demonstrated intermittent staining patterns along neurites which are similar to the periodic pattern seen in our injury model[4]. We

Fig. 10. Taxol prevention of H_2O_2-induced microtubule disruption. Fluorescence micrographs of PC12 cells stained with a double antibody technique for tubulin were obtained as described in methods. A: cells 2 h after exposure to 100 μM H_2O_2. Note the characteristic pattern of microtubule disruption in the neurites of the injured cells. B: cells after 2 h of treatment with 5 μM Taxol and 100 μM H_2O_2. Note the presence of intact neurites with a continuous staining pattern for tubulin. This effect of Taxol was also seen at 4 and 6 h after oxidant exposure in > 90% of cells examined. C: uninjured controls exposed for 2 h to 5 μM Taxol. Note the presence of intact neurites with a continuous pattern of tubulin staining.
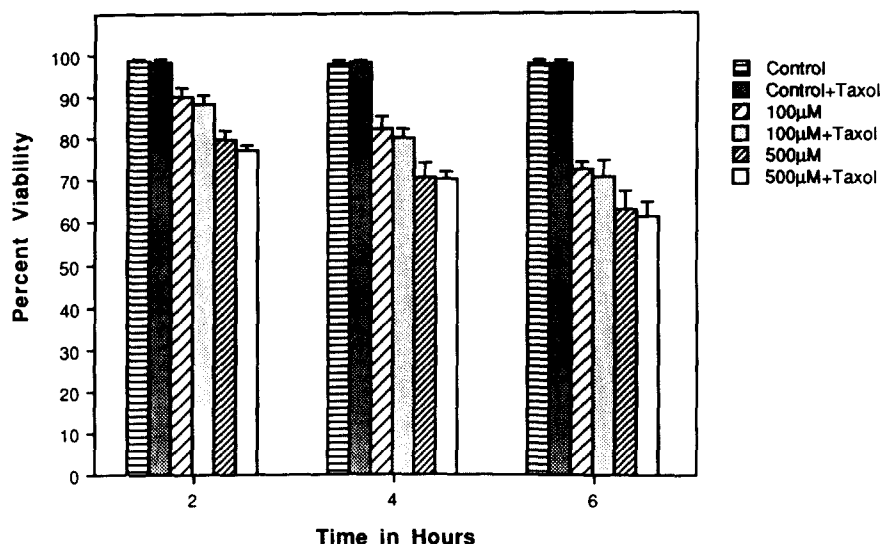
Fig. 11. The effect of microtubule stabilization with 5 μM Taxol on PC 12 cell viability following H_2O_2 exposure. Each bar represents the mean + S.D. of $n = 5$ separate determinations. 100 μM = 100 μM H_2O_2, 500 μM = 500 μM H_2O_2; Taxol = 5 μM Taxol.

have examined the staining pattern of microtubules in differentiated PC 12 neurites stained with the monoclonal antibody 6-11-B-1 which stains acetylated α-tubulin and found that it produces a uniform staining pattern identical to staining with the monoclonal anti β-tubulin, indicating that acetylated α-tubulin is uniformly distributed in the microtubules of PC 12 neurites (data not shown). This uniform pattern of staining[1] is in contrast to the discontinuous pattern of distribution of acetylated α-tubulin seen in cerebellar granule cell cultures stained with the monoclonal antibody 6-11-B1[4]. Other factors which may play a role in determining the pattern of tubulin depolymerization seen in these experiments include the microtubule-as-

sociated proteins called STOPs (stable-tubule only polypeptides)[21]. One of these, a 145-kDa protein has been localized to cold stable microtubules in neurons[27]. Periodic distribution of such proteins along neuritic microtubules might define regions which are relatively resistant to cold or Ca^{2+}-induced tubulin depolymerization.

Differences in the rate of cell lysis induced by low micromolar concentrations of H_2O_2 seen between PC 12 cells in comparison with earlier studies of endothelial cells cannot be accounted for on the basis of differences in rates of catalase-dependent H_2O_2 consumption by the cells. It is interesting to note that in these experiments aminotriazole-inhibitable H_2O_2
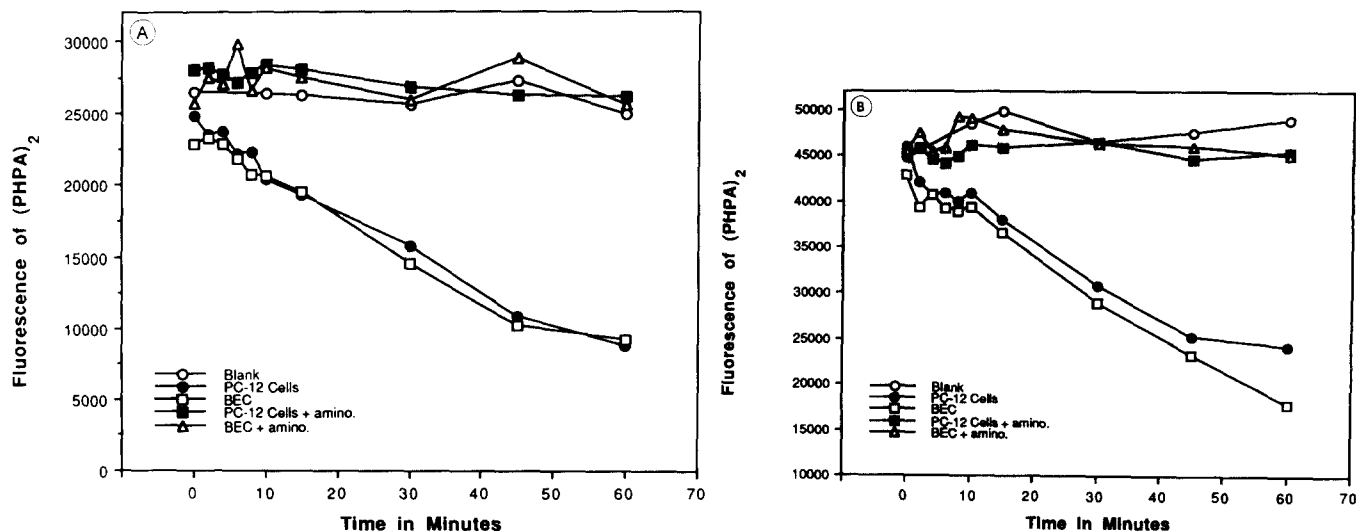

Fig. 12. H_2O_2 consumption by adherent PC 12 and endothelial cells. A: comparison of the consumption of a 100 μM bolus of H_2O_2 by adherent PC 12 and endothelial cells (amino = 12 mM 3-amino-1,2,4-triazole). Each data point represents the mean of $n = 4$–6 separate determinations. B: comparison of the consumption of a 1 mM bolus of H_2O_2 by adherent PC 12 and endothelial cells (amino = 12 mM 3-amino-1,2,4-triazole). Each data point represents the mean of $n = 4$–6 separate determinations.

consumption accounted for essentially all H_2O_2 consumption by both cell types at high and low H_2O_2 concentrations. In previous work we found that endothelial cells are quite resistant to H_2O_2-mediated disruption of $[Ca^{2+}]_i$ homeostasis, requiring millimolar concentrations of the oxidant to induce significant elevation of $[Ca^{2+}]_i$[10]. If our modelling of the H_2O_2-mediated PC 12 injury with ionomycin is correct, the differentiated PC 12 cells may be quite vulnerable to oxidant-mediated disruption of $[Ca^{2+}]_i$ homeostasis and this difference may account for the more rapid kinetics of cell lysis seen with these cells. This would also be consistent with the important role elevation of $[Ca^{2+}]_i$ is thought to play in the action of other neurotoxins[18]. A recent study of H_2O_2-mediated injury to undifferentiated PC 12 cells demonstrated that low micromolar concentrations of H_2O_2 could increase $[Ca^{2+}]_i$ in the cells[7].

In summary, PC 12 cells differentiated to a neuronal phenotype with NGF treatment provide an excellent cellular model for examination of the toxic effects of oxidants on neurons. PC 12 neurites and particularly neuritic microtubules are early targets of H_2O_2 injury, and this injury appears to be Ca^{2+}-dependent.

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