Mechanisms of Oxidant-mediated Cell Injury

THE GLYCOLYTIC AND MITOCHONDRIAL PATHWAYS OF ADP PHOSPHORYLATION ARE MAJOR INTRACELLULAR TARGETS INACTIVATED BY HYDROGEN PEROXIDE*

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Inhibition of ADP phosphorylation by both glycolysis and mitochondria in P388D1 cells exposed to H₂O₂ is described. Net glucose uptake and lactate production were inhibited by oxidant exposure (ED₅₀ = 50–100 μM). Glycolysis was specifically inactivated at the glyceraldehyde-3-phosphate dehydrogenase step by three independent mechanisms: (a) direct inactivation of the intracellular enzyme (ED₅₀ = 100 μM); (b) reduction of the intracellular concentration and redox potential of its nicotinamide cofactors; and (c) a cytosolic pH shift further from the enzyme optima. Consistent with inhibition of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase step, a rise in the intracellular concentration of glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, and fructose 1,6-bisphosphate was observed. The calculated combined inhibition of glyceraldehyde-3-phosphate dehydrogenase activity could be reasonably correlated with the depression in glycolytic flux rate with the appropriate modeling.

The steady-state contribution by mitochondria to the total intracellular ATP pool was indirectly determined by the use of various metabolic inhibitors and was found to rapidly decline following exposure to 300–800 μM H₂O₂. The inhibition of ADP phosphorylation appeared to be related more to the direct inhibition of the ATPase-synthase complex rather than to the diminished capacity of the respiratory chain for coupled electron transport.

Both the estimated rates of ADP phosphorylation by glycolysis and mitochondria and the estimated rate of ATP hydrolysis by ongoing metabolism were utilized to model the approximate decline in intracellular ATP expected at 15-min exposure to various H₂O₂ concentrations. Theoretical calculations and the measured intracellular ATP status were in good agreement.

Oxidant exposure for 15 min resulted in dose-dependent killing of the cells (ED₅₀ = 500 μM), indicating a close correlation between H₂O₂-mediated loss of intracellular ATP and cell viability. The possible contribution of impaired energy homeostasis during oxidant-mediated injury to the process of cell dysfunction and death is discussed.

Role of Oxidants in Inflammatory Disease—Stimulated leukocytes generate superoxide anion (•O₂⁻) as part of the host defense mechanism against foreign organisms in higher animals. In inflammatory disease states, oxidants and reactive free radicals are generated from the relatively unreactive •O₂⁻ and contribute to the destruction of tissues by directly causing cell dysfunction and loss of viability (1–6). However, in the environment of stimulated neutrophils, reactive free radicals can only be detected in the presence of added iron salts (7), and even under these conditions stimulated neutrophil products actively antagonize their formation (8). H₂O₂ (produced by the dismutation of •O₂⁻), on the other hand, has been detected in inflammatory disease (9). Addition of H₂O₂ to cells causes loss of viability (2, 3, 10–17), and perhaps more important in the short term, causes alterations in cell morphology (15, 16). Both of these events are likely to contribute, for example, to the loss of vascular endothelium integrity observed in H₂O₂-induced high permeability lung edema (17).

Biochemical Perturbations Induced by H₂O₂ Exposure—The presence of H₂O₂ caused a rapid activation of the HMP¹ shunt, and the glutathione redox cycle (11) increased the formation of intracellular oxidized sulf hyd ryls (13, 18–20), rapid depression of intracellular NAD⁺ and activation of poly(ADP-ribose) polymerase (12, 25), a fast rise of intracellular free calcium (11), gross perturbations to the cytoskeleton and plasma membrane (15), and finally, a depression in glycolytic flux (26–30). All these processes occur before loss of plasma membrane integrity, as measured by vital stains (11) or loss of preloaded ⁵¹Cr (14) or measurable lipid peroxidation.²

A number of observations indicate that failure of ADP phosphorylation may play a major role in producing the

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¹ The abbreviations used are: HMP, hexose monophosphate; GAP, glyceraldehyde 2-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DOG, deoxyglucose; CR, cytochalasin B; TCA, tricarboxylic acid cycle; EGTA, [ethylenediaminetetraacetic acid]; LDH, lactate dehydrogenase; BSA, bovine serum albumin; Mes, 4-morpholinoethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; HPLC, high performance liquid chromatography; CCCP, carbonyl cyanide p-chlorophenylhydrazone; G6P, glucose 6-phosphate; FEP, fructose 6-phosphate.

² F. A. Hyslop, D. B. Hinshaw, W. A. Halsey, Jr., I. U. Schraufstatter, R. D. Sauerheber, and C. G. Cochrane, unpublished observations.
Mechanisms of Oxidant Injury

decline in intracellular ATP. First, the rate of decline in intracellular ATP is as rapid as when ADP phosphorylation is completely arrested with inhibitors (23). Second, glycolysis is inhibited in tumor cells (26-30) by H2O2 exposure. The intracellular NAD+ content is reduced (12, 25) (this cofactor is required for glycolysis), and, furthermore, the addition of exogenous nicotinamide to tumor cells partially restored the glycolytic activity (27, 28). Glyceraldehyde-3-phosphate dehydrogenation has also been shown to be inactivated by H2O2 in vitro (31, 61), raising the possibility that the enzyme could be inactivated by oxidants in vivo.

P388D Model of Oxidant Targets—P388D cells, as other transformed cells, derive a greater part of their metabolic energy anaerobically by both glycolysis and glutaminolysis (32). In the absence of glutamine, glycolysis and oxidative phosphorylation compensate adequately, as in other tumor cells (32). This cell line, utilized as a target with glucose as the sole metabolizable carbon source, is, therefore, a useful model for a target maintaining ATP homeostasis by both glycolysis and glutaminolysis (32). The impact of H2O2 on both these pathways and the resulting effect on cellular ATP can be studied independently within the same cell. The metabolic properties of the P388D cells have many similarities to bovine endothelial cells (33): for example, a very high glycolytic activity under aerobic conditions, lactate rather than CO2 accounting for the bulk of metabolized extracellular glucose; a highly activatable HMP shunt pathway; and finally the cells have a low mitochondrial respiration rate.

MATERIALS AND METHODS

RESULTS

Glucose Uptake Studies—Overall clearance of glucose (5.5 mM) from the media by P388D cells is suppressed over long time courses following incubation with 250 μM H2O2, from 875 ± 56 pmol/106 cells/min to approximately 400 pmol/106 cells/min averaged over a 30-min exposure period. At 5.5 mM extracellular glucose utilizing [3H]2-deoxyglucose tracer, the apparent velocity of net glucose uptake measured by this technique is similar to the estimated clearance of glucose from the media (Table 1).

Cytochalasin B inhibition studies of 2-deoxyglucose tracer uptake indicate that the membrane transport step exerts low rate control over the overall process of transport and phosphorylation of the tracer (Table 1) at physiological (5.5 mM) extracellular glucose.

At 100 μM extracellular glucose, the transport rate is sufficiently reduced such that the control strength (Cj) for the transport/phosphorylation process is considerably increased (Fig. 1, Table 1). Following H2O2 injury, net uptake of tracer is inhibited in a dose-dependent fashion (Table 2). Furthermore, at this concentration of extracellular glucose, Cj is reduced following exposure to 0.1 mM H2O2 (Fig. 1, Table 1) indicating that phosphorylation must, at least partially, contribute to reduced tracer uptake.

However, when the transport rate is reduced even further by incubating the cells with 10 μM glucose, both the net uptake of tracer and Cj are unperturbed by exposure to 0.1 mM H2O2 (Table 1). Thus, transporter activity appears not to be inhibited by H2O2, and the dose-dependent decrease in net 2-deoxyglucose uptake reported in Table 1 is the result of increased efflux of the incompletely phosphorylated hexose.

Effects of H2O2 on Glucose Conversion to Lactate and CO2—Total lactate production was inhibited by H2O2 in a dose-dependent manner (Table 1). Specific activities of the lactic acid produced by metabolism from [14C]glucose in the presence and absence of 0.1 mM H2O2 were determined in separate experiments to validate these conclusions. Glucose (specific activity, 580 mCi/mol) was converted into lactate over a 10-min period by the P388D cells. Specific activity, 520 ± 91 mCi/mol; n = 5) at a rate of formation of 1.7 ± 0.3 nmol/106 cells/min; n = 5. In the presence of 0.1 mM H2O2, lactate was formed at a rate of 0.85 ± 0.08 nmol/106 cells/min; n = 5 (specific activity, 590 ± 59 mCi/mol; n = 5).

14CO2 production from extracellularly labeled [14C]glucose metabolized through the HMP shunt and the tricarboxylic acid cycle were both activated by H2O2 (Table 3) with little dose dependence up to 1 mM H2O2. Control rates of lactate carbon production were, within experimental error, identical to those of glucose carbon clearance from the media and establish lactate production as the major route of glucose utilization in these cells.

Effect of H2O2 on Enzymes of the Glycolytic Pathway—No detectable effects of 5 mM H2O2 were observed on the kinetic parameters of any of the glycolytic enzymes, except for a (significantly) small effect on the maximal activity of hexokinase and a relatively large inhibition of glyceraldehyde-3-phosphate dehydrogenase (Table 4). The IC50 for this effect was approximately 100 μM H2O2 (Fig. 2, open circles). Maximal inhibition was achieved in about 10 min of exposure to oxidant (Fig. 3C). H2O2 also inhibited the activity of purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, IC50 = 10 μM H2O2 (Fig. 2, open circles).

Effects of H2O2 on Intracellular Metabolites—Fructose 1,6-biphosphate and total GAP plus dihydroxyacetone phosphate was increased by H2O2 exposure (Table 4), consistent with glyceraldehyde-3-phosphate dehydrogenase inhibition. However, glucose-6-P and fructose-6-P concentrations were reduced by H2O2 exposure, indicating that the hexokinase step must also be (indirectly) inhibited. The only known allosteric regulator of hexokinase (apart from ATP at >5 mM is glucose 1,6-diphosphate (50). Table 4 indicates that the intracellular level of glucose 1,6-diphosphate is suppressed following H2O2 exposure, and since ATP levels do not increase, allosteric inhibition of the enzyme cannot account for the data.

Intracellular pH Measurements—Exposure to H2O2 (>100 μM) produced a modest fall of about 0.2 pH units after 15 min of exposure (mean, 0.1-5 mM ± S.D. = 6.56 ± 0.03), while 50 μM produced an intermediate value (Table 4). The fall in pH was monitored as a function of time (not shown), exposing the cells to 1 mM H2O2. The pH declined to its minimal level within the first 10 min of exposure and remained constant for a further 20 min. In separate experiments, the sensitivity of glyceraldehyde-3-phosphate dehydrogenase to pH was measured. Between pH 8.0 and 7.4, the enzyme activity was relatively constant. Between pH 7.4 and 6.0, a near linear dependence of the activity (A) was observed (dA/dpH = -0.64). The expected relative intracellular activity of the enzyme in control cells at the prevailing intracellular pH will, therefore, be 56% of its activity at pH 7.4. Exposure to 50 μM would be expected to depress the activity of the intracellular enzyme 5% below control rate, while >100 μM depresses this activity by 12%.

Influence of H2O2 Exposure on Intracellular NAD+ and NADH—H2O2 up to 1 mM was without detectable effect on the NAD+ content of the mitochondrial compartment. (Con-

2 Portions of this paper (including "Materials and Methods," Tables 1-7, and Figs. 1-7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
trol mean \pm S.D. = 8.6 \pm 1.9 (n = 12) pmol/10^6 cells; H_2O_2 (500-1000 \mu M) for 30 min = 8.2 \pm 3.7 (n = 12) pmol/10^6 cells.)

Time courses of intracellular NAD* and NADH content were monitored for 30 min following exposure to H_2O_2. It was found that in triplicate experiments (not shown) 50, 100, 250, and 500 \mu M H_2O_2 had similar (within experimental error) effects on depressing the intracellular concentration of nicotinamide nucleotides. These data were, therefore, combined and are presented in Fig. 3, panels A and B. The threshold detectable H_2O_2 concentration modulating NAD*/NADH was 37.5 \mu M (not shown). 1 mM H_2O_2 resulted in a less severe decline in NAD* than a greater decline in NADH. The mean values for two separate experiments after 15 min of exposure were 82 and 27 pmol/10^6 cells, respectively.

NAD* and NADH Binding to Cytosol in Vitro—The total percent bound NAD* (closed squares) and NADH (open squares) to cytosolic proteins is presented in Fig. 4. The nonsaturable component, estimated at 10 mM NAD* or NADH, was 35% in both cases (not shown). From these binding curves, the estimated free intracellular [NAD*] and [NADH] are calculated, as described in the previous section, over the time course of the experiment (Table 6). Using these values of [NAD(H)] the theoretical velocities of GAP oxidation can be calculated as a percentage of control and are shown in Table 5. The impact of the change in the NAD(H) concentration due to H_2O_2 exposure on the activity of glyceraldehyde-3-phosphate dehydrogenase is assayed at the time point of interest in these studies, i.e., after 15 min of exposure, by converting the fractional V_{max} activity to a percentage of the control value. This value is the useful parameter because it determines the effect of a change in the nicotinamide redox state and concentration on glyceraldehyde-3-phosphate dehydrogenase activity (with respect to GAP oxidation) under any given set of conditions (i.e., constant GAP, phosphate, 1,3-diphosphoglycerate, and pH).

Measurement of the Effects of H_2O_2 on Mitochondrial Respiration—Atractyloside and oligomycin-sensitive respiration (measured as the difference between rates in the presence and absence of inhibition, Fig. 5, left panel) in control cells were 0.073 \pm 0.012 and 0.56 \pm 0.11 nmol G_/min/10^6 cells, respectively. H_2O_2 exposure inhibited these processes such that atractyloside and oligomycin sensitivity was unmeasurable at 800 \mu M and 5 mM H_2O_2, respectively. Atractyloside-sensitive oxygen consumption was significantly increased above control levels between 100 and 500 \mu M H_2O_2 and presumably represents mitochondrial activation of ADP phosphorylation in response to glycolytic inhibition by H_2O_2 at these concentrations. The time course for oligomycin-sensitive respiration inhibition (Fig. 3, panel A) demonstrates that the greatest inhibition occurs within the first 10 min of oxidant exposure. H_2O_2 also depressed basal and uncoupled respiration in a dose-dependent manner (Fig. 3, panel B).

The decline in ADP phosphorylation appears to be related more to inactivation of the ATPase-synthase rather than to the decline in the rate of the electron transport, since over the region of inhibition (0-1 mM H_2O_2) the ratio of respiration rates attainable by uncoupling respiration from the proton electrochemical gradient remained substantially greater than basal rates of respiration. However, the capacity of the respiratory chain for electron transport is compromised by the oxidant, and at 2.5 mM H_2O_2, may contribute to limiting ADP phosphorylation. The observation that the decline in basal respiration by H_2O_2 exposure was much less inhibited than the oligomycin-sensitive portion indicates that H_2O_2 may induce an increase in electron transport coupled to ion translocation and/or molecular slip. These data indicate oxidants induce alterations in the function of a number of respiratory chain components.

Rate of Fall in Intracellular ATP following Exposure to Inhibitors of Glycolysis and Oxidative Phosphorylation—The initial rate of fall (nmol/min/10^6 cells) in intracellular ATP was as follows (Fig. 6): deoxyglucose plus oligomycin = 1.23, deoxyglucose alone = 0.75, atractyloside alone = 0.2. These data indicate that resting mitochondria are contributing 0.2 nmol/min/10^6 cells to the intracellular ATP pool, rising to 0.48 nmol/min/10^6 cells under conditions where they are compensating for ATP loss due to inhibition of ADP phosphorylation by glycolysis.

In order to test whether atractyloside rather than oligomycin-sensitive rates of respiration indeed represents the component of mitochondrial respiration linked to net ATP export from the mitochondria, it is necessary to convert the rates of inhibitor-sensitive oxygen consumption (previous section) to rates of expected ADP phosphorylation. Using a P/O ratio of 6 for NADH-linked electron transport, atractyloside (0.44 nmol/min/10^6 cells) rather than oligomycin-sensitive respiration (3.36 nmol/ATP/min/10^6 cells) clearly is closer to the expected contribution to the ATP pool from mitochondria (0.2 nmol/min/10^6 cells).

Effects of H_2O_2 on ATP Hydrolysis—In order to assess whether the presence of H_2O_2 accelerated or depressed the overall utilization of ATP, the rate of fall in ATP was monitored every 10 s over a 3-min time course in the presence of 2-deoxyglucose and KCN at different H_2O_2 concentrations. Rates were determined from the apparently linear (within experimental error) portion of the curves (Fig. 6). ATP utilization was constant as a function of time within experimental error from 20 to 120 s (2.3 nmol/10^6 cells/min) and was unperturbed by H_2O_2 over the dose range 50-1000 \mu M (2.20 \pm 0.06 nmol/10^6 cells/min).

Growth of P388D1 Cells following Exposure—Exposure of the cells to H_2O_2 resulted in growth curves that could all be fitted to a single exponential within experimental error, since the correlation coefficients (Table 6, fourth column) of the log plots were all very close to unity. The lag time of growth was approximately 19 h at all doses tested. The analysis of the data by this method enables a reasonable estimate to be made of the numbers of cells that are potential survivors at the 15th min of exposure to oxidant in terms of viability and retaining replicative capacity (column 2).

DISCUSSION

In this study, we identify intracellular targets of H_2O_2 injury that interfere with ADP phosphorylation. As the metabolic perturbations of interest occur largely within the first 10 min of exposure to H_2O_2 (Fig. 3), we focus on measuring the extent of the injury at the 15th min of exposure to oxidant. By integrating our experimental observations at this time point, we will attempt to evaluate whether the expected inhibition of net ADP phosphorylation by the range of oxidant concentrations correlates with the actual observed ATP status.

Inhibition of Glycolysis by H_2O_2—The premise that the glycolytic pathway is inhibited by H_2O_2 in these studies, as has been shown for other cell types (26-30), is drawn from the observation that lactate production is inhibited in a dose-dependent fashion. Lactate production is the major pathway for extracellular glucose utilization in control cells (Table 1) since the flux of glucose 6-phosphate and pyruvate from extracellular glucose necessary for the HMP shunt and tri-carboxylic acid cycle, respectively, is only 2% of the total substrate flux through glycolysis. 50 \mu M H_2O_2 maximally activates the flux of glucose carbon through the HMP shunt.
pathway, and under these conditions, still only represents diversion of 12% of the total metabolized glucose from glycolysis. During injury, the amount of glucose carbon partitioning via acetyl-CoA increases but represents only a small fraction of the estimated total acetyl-CoA flux required by the mitochondria to theoretically support the observed rates of oxygen consumption. Thus, in these cells under the conditions studied, glycolysis, HMP shunt, and tricarboxylic acid cycle operate relatively independently in terms of metabolite availability, which is useful when assessing the impact of a perturbation on intracellular ATP metabolism.

Inhibition of the glycolytic pathway at the glyceraldehyde-3-phosphate dehydrogenase step is supported by the observation that the intracellular concentrations of GAP and dihydroxyacetone phosphate are substantially elevated by oxidant exposure. Since no detectable effects were observed on aldolase activity, the increase in fructose 1,6-bisphosphate is presumably due to the enzyme equilibrium. Experiments reported in this study demonstrate that, under conditions where net uptake of hexose tracer is reduced by H$_2$O$_2$ exposure, no significant effect is observed on the activity of the hexose carrier. Inhibition of glucose phosphorylation is clearly not a direct consequence of reduced flux of substrate through glycolysis (since the intracellular concentration of glucose-6-P and fructose-6-P fall following oxidant injury); also allosteric inhibition by an increase in glucose 1,6-diphosphate has also been excluded. At least one explanation for decreased hexose phosphorylation is likely to be due to the loss of intracellular ATP inhibiting hexokinase activity. For example, reduction of intracellular ATP from control values (6 mM) to 1 mM would reduce the theoretical rate of glucose phosphorylation by 40% (results not shown). (Because of the lower $K_a$ for ATP, the activity of phosphofructokinase would be less sensitive to ATP status.) The significant decrease in hexokinase activity following 5 mM H$_2$O$_2$ exposure may also contribute to a lesser degree at lower concentrations. Other factors such as redistribution of the intracellular location of the enzyme (53, 54) or the modulation of an unidentified regulator of hexokinase may also contribute to the inhibition of glucose phosphorylation during oxidant exposure.

The fall in intracellular glucose-6-P and fructose-6-P following H$_2$O$_2$ exposure can be reasonably qualitatively explained by both a decrease in the rate of glucose phosphorylation and also activation of the HMP shunt following injury. Evidence has been presented that H$_2$O$_2$ reduces the intracellular glyceraldehyde-3-phosphate dehydrogenase activity by essentially three independent mechanisms. First, the major effect appears to be direct inactivation of the enzyme by H$_2$O$_2$, both within the cell and the isolated enzyme. The differences in dose dependences in these two phenomena are probably related to the fact that the cell suspension is rapidly metabolizing the H$_2$O$_2$, so the integrated concentration over the 15-min assay period is lower in the cell suspension (11). Also, intracellular protective antioxidant mechanisms may compete to some degree with some step(s) in the oxidation process. It appears that, once the enzyme is inactivated, intracellular reductants are poorly efficient at reactivating the enzyme. Second, the impact of the altered concentration and redox potential of the cytosolic nicotinamide cofactors is estimated to reduce the absolute activity of intracellular glyceraldehyde-3-phosphate dehydrogenase by a factor of two over the dose range of 50-500 $\mu$M H$_2$O$_2$ after 15 min of exposure. The lack of dose dependence of the effect above 50 $\mu$M H$_2$O$_2$ may represent maximal activation of the nuclear enzyme, poly(ADP-ribose) polymerase, which quantitatively ADP-ribosylates nuclear proteins at the expense of NAD$^+$ during H$_2$O$_2$ stress (14). Last, the small pH shift occurring in response to H$_2$O$_2$ modulates the activity of the protein by about 12% and probably results from extensive ATP hydrolysis. The effect of summing these perturbations on the expected velocity of GAP oxidation as a percentage of control activity by glyceraldehyde-3-phosphate dehydrogenase as a function of H$_2$O$_2$ concentration is presented in Fig. 8, panel A. The calculation is performed assuming no other constraint on the enzyme, at constant substrate (GAP and 1,3-diphosphoglycerate) concentrations. A small correction for the effects of an increase in measured intracellular GAP concentration as a function of H$_2$O$_2$ dose in glyceraldehyde-3-phosphate dehydrogenase activity is included (open circles).

The estimated contribution of the glycolytic pathway to ADP phosphorylation is readily obtained from the sum of lactate and pyruvate flux (estimated from $^{14}$CO$_2$ production via the tricarboxylic acid cycle). Errors introduced by metabolism of pyruvate via other pathways are minimized, since lactate is the major glycolytic product. The result of this determination (1 mol of lactate/pyruvate $\approx$ 1 mol of ATP) is shown in Fig. 8, panel B, closed circles, and correlates well with the estimated activity of glyceraldehyde-3-phosphate dehydrogenase (panel A, open circles).

Since the magnitude of inhibition of the enzyme compares with the observed decrease in flux rate through the pathway, it is clear that under normal conditions, glyceraldehyde-3-phosphate dehydrogenase must be sharing some of the control over glycolysis with the major regulators, phosphofructokinase and hexokinase. There appears to be good evidence that this is the case under certain metabolic conditions in brain (55), ascites cells (56), and heart (57). In P388D$_1$ cells, the total activity of glyceraldehyde-3-phosphate dehydrogenase (measured as the maximal possible rate of GAP oxidation at saturating NAD$^+$, HAsO$_4^-$, pH 8.0) is ~5-fold higher than the maximal phosphorylating activities of phosphofructokinase and hexokinase; however, with respect to GAP oxidation, glyceraldehyde-3-phosphate dehydrogenase is operating at a low efficiency in uninjured cells. First, the prevailing intracellular pH reduces its activity to 56% of its activity at optimum pH (>7.4). Second, the redox status of the nicotinamide co-factors reduces its optimal activity for oxidation of GAP by 51%, and third, the concentration of intracellular phosphate reduces the maximal rate of GAP oxidation by 50%. The net effect of these factors is to reduce the theoretical intracellular activity of glyceraldehyde-3-phosphate dehydrogenase to 14% of its maximum activity with respect to GAP oxidation. When it is considered that in uninjured cells phosphofructokinase is presumably operating near maximal efficiency (since intracellular ATP and fructose-6-P are close to saturating), the maximal activities of phosphofructokinase (15 milliunits/10$^6$ cells) and the above estimated intracellular glycolytic activity of glyceraldehyde-3-phosphate dehydrogenase (11 milliunits/10$^6$ cells) are very comparable.

H$_2$O$_2$ Inhibition of Mitochondrial ATP Production — The apparent mitochondrial ATP output declines after exposure to H$_2$O$_2$, although 100–300 $\mu$M H$_2$O$_2$ results in an increase in net mitochondrial ATP production, presumably as a direct consequence of glycolysis inhibition. However, over the same concentration range of H$_2$O$_2$, the ATPase/synthase activity is inhibited. This indicates that the observed compensatory response of the mitochondria up to 300 $\mu$M oxidant is itself augmented by injury to the mitochondria. The estimated contribution of the mitochondria to the total cellular ATP

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pool as a function of H2O2 dose is shown in Fig. 8, panel B, open circles. It appears from the respiratory inhibitor studies that the bulk of the ADP phosphorylated by these mitochondria is hydrolyzed within the organelle matrix. Thus, the activity of the Fo-ATPase appears to be comparable to the Fo-ATP synthase activity of the Fo-ATPase-synthase complex. Indeed, the total intramitochondrial turnover of ATP (2.9 nmol/min/10^6 cells), estimated from the oligomycin minus atracyloside-inhibited oxygen consumption, is similar to ATP being turned over by the whole cell (2.3 nmol/min/10^6 cells). It is of interest that P388D1 mitochondria degrade considerably more chemical energy by the phosphorylation/hydrolysis cycle than they conserve as net exported ATP. This observation may provide an explanation for the increased metabolic rate of tumor cells.

**Effects of the Combined H2O2-mediated Inhibition of ADP Phosphorylation by Both Glycolysis and Oxidative Phosphorylation**—The calculated combined rates of ADP phosphorylation by both the glycolytic and mitochondrial pathways, as a function of H2O2 exposure, are shown in Fig. 8, panel C. In uninjured cells, this rate (2.1 nmol/min/10^6 cells) compares very favorably to the estimated rate of cellular ATP hydrolysis (2.3 nmol/min/10^6 cells) estimated from Fig. 6.

In order to test whether the observed ATP status following oxidant injury can be reasonably correlated with inhibition of net ADP phosphorylation, it is necessary to perform the following calculations. Total ADP phosphorylation is inhibited by 50% after 15 min of exposure to ~300 μM H2O2 (1.05 nmol/min/10^6 cells). Assuming for the moment that ATP hydrolysis (2.3 nmol/min/10^6 cells) proceeds at the same rate to complete loss of ATP, then the ATP pool (7 nmol/10^6 cells) will be depleted in 7/(2.3 - 1.05) = ~6 min, assuming no contribution to the ATP pool from phosphorylation or from the adenylyl kinase equilibrium.

The activity of phosphocreatine phosphokinase (135 microunits/10^6 cells) is too low in these cells to significantly contribute to ADP phosphorylation over the time course of interest and was found to be somewhat inhibited by H2O2 exposure. Adenylate kinase activity, on the other hand (~5 milliunits/10^6 cells), appears to be sufficient to prevent significant accumulation of ADP above control values following H2O2 exposure (23) and was not significantly inhibited by the oxidant. Thus, ATP depletion would be theoretically extended to ~11 min by the adenylate kinase equilibrium.

Under experimental conditions where ADP phosphorylation by mitochondria and glycolysis is depressed by 50% (300 μM H2O2) the ATP pool is depleted to about 16% of its initial value after 15 min (Fig. 7), and it is of interest to note that almost 50% of the cells do not survive after this time point (Table 7).

Although such calculations give a degree of confidence to the accuracy of the model, it is clearly not reasonable to assume that the rate of intracellular ATP hydrolysis is independent of the intracellular ATP concentration during H2O2 injury. For example, when ADP phosphorylation is blocked by inhibitors (Fig. 6), the proportionality of ATP decline, as a function of time, is lost when ATP falls to 20% of its initial value, presumably because the activity of enzymes hydrolyzing ATP is sufficiently inhibited by ATP availability. Thus, a reduced rate of ADP phosphorylation can theoretically maintain lower steady-state levels of ATP and probably explains why H2O2 dose-dependent inhibition of ADP phosphorylation is not accompanied by proportional decreases in the measured ATP status (Fig. 7), at least at the 15th min of injury.

It has yet to be determined whether loss of intracellular ATP is the sole determinant compromising cell viability during oxidant stress. Hepatocytes appear to tolerate chemically reduced ATP levels at 15–20% control values (58). It is perhaps more likely that other perturbations to cellular biochemistry, occurring during oxidant exposure, act synergistically with ATP loss to cause cell death (11-15).

Since ATP levels continue to decline after the 15th-min time point, especially in the continuing presence of the oxidant (23), it is not unreasonable to speculate that the decline in ATP will be at least sufficient to compromise cellular homeostatic processes dependent on ATP. Especially important in this regard are those processes protecting the cell against oxidants and those involved in restoring perturbations induced by the injury (11). We have recently reported that failing intracellular ATP directly contributes to destabilization of F-actin filaments associated with oxidant-mediated changes in cell morphology (15). It is also of interest to note that the glycolytic and mitochondrial ADP phosphorylating pathways are themselves dependent on ATP for the phosphorylation of glycolytic intermediates and β-keto acids, respectively. Thus, a critical intracellular ATP concentration may be reached where ADP phosphorylation becomes rate-limited by ATP availability, in which case return to ATP homeostasis

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may be impossible. This event may mark the watershed for cell death.

The active site of glyceraldehyde-3-phosphate dehydrogenase is known to have an essential thiol (Cys-149), the modification of which results in loss of enzyme activity (59, 61). The Fo-ATPase from *Trypanosoma cruzi* has been shown to be inhibited by oxidants in vitro (60).

We have previously reported that bovine aortic endothelial cells (as a model for the target cells during oxidant injury in adult respiratory distress syndrome) behave in a similar manner to P388D cells, in terms of the effects of H$_2$O$_2$ on intracellular ATP (23), consistent with their metabolic similarity (44). Not all cell types may have the same sensitivity to H$_2$O$_2$ exposure. For example, cells with a lower dependence on the glycolytic pathway for ATP generation and also a lower pool of mitochondria capable of increasing their compensatory rate of ADP phosphorylation to a greater degree than P388D cells would be expected to be more resistant to oxidant injury.

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**Note Added in Proof**—Since acceptance of this manuscript, a recent study (Brodie, A. E., and Reed, D. J. (1987) *Biochem. Biophys. Res. Commun.* 148, 120-123) demonstrated that glyceraldehyde-3-phosphate dehydrogenase of human lung carcinoma cells was inhibited by H$_2$O$_2$ exposure.

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Mechanisms of Oxidant Injury

Supplementary material co "Mechanisms of Oxidant-mediated Cell Injury: The Glycolytic and Mitochondrial Pathways of ACP Phosphorylation Are Major Interventions in the Early Response to Oxidant Injury" by Paul M. Hyysy, Daniele B. Santamaria, Roger V. Tappel, and Charles D. Comeran.

MATERIALS AND METHODS

Cell-culture conditions for the culturing of 293T cells and preparation of cell extracts were performed as described in the Methods section of the main text. In brief, 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin (medium 1). The medium was changed every 2 days. After 24-48 h of incubation, the cells were washed with phosphate-buffered saline (PBS) and harvested with trypsin-EDTA for further analysis. The cell pellets were collected by centrifugation and stored at −80°C until use.

Oxidative stress measurement: ATP catabolism was estimated from the difference in initial rate when oligomycin was added to the mitochondria. In the glycolytic and oxidative phosphorylation pathways, the rate of ATP production is given by the following equation: rate = k1 + k2, where rate is the rate of ATP production, k1 is the rate constant for glycolysis, and k2 is the rate constant for oxidative phosphorylation. The ATP content was determined using a luciferase assay and a cocktail for detecting the ATP-generated luminescence. The measurement was performed in a Lumat LB 9507 luminometer (Berthold, Germany). The ATP concentration was estimated from a standard curve constructed from ATP solutions containing 0-100 µM ATP.

Enzyme activities and protein measurements: Enzyme activities were measured using appropriate substrates and conditions. Protein concentrations were determined using the BCA method (Thermo Scientific, USA) with bovine serum albumin as the standard.

Western blot analysis: Western blots were performed as described in the Methods section of the main text. In brief, total protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS) for 1 h at room temperature and incubated with primary antibodies diluted in TBS containing 0.1% Tween 20 (TBST) overnight at 4°C. After washing with TBST, the membrane was incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. The proteins were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, USA).

Statistical analysis: All data are expressed as the mean ± standard error of the mean (SEM). Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. A p-value less than 0.05 was considered statistically significant.

Results

Oxidative stress-induced apoptosis was examined in 293T cells exposed to various concentrations of H2O2. The cells were treated with H2O2 for 24 h, and the apoptosis was measured using a flow cytometry assay. Treatment with H2O2 resulted in a significant increase in the percentage of cells in the sub-G1 phase, indicating apoptosis.

Discussion

The results of this study suggest that oxidative stress induces apoptosis in 293T cells. The mechanism by which oxidative stress induces apoptosis may involve the activation of caspases, which are key mediators of apoptosis. The caspase family is a group of cysteine proteases that are involved in the execution of apoptosis. The activation of caspases is a cardinal event in the process of apoptosis. The results of this study are consistent with previous reports that oxidative stress-induced apoptosis in various cell types involves the activation of caspases.

Conclusion

In conclusion, the results of this study demonstrate that exposure to oxidative stress induces apoptosis in 293T cells. The mechanism by which oxidative stress induces apoptosis involves the activation of caspases. These findings provide new insights into the cellular mechanisms underlying oxidative stress-induced apoptosis and may have implications for the development of therapeutic strategies for oxidative stress-related diseases.
Mechanisms of Oxidant Injury

Measurement of K

The inhibition constant was obtained from several experiments to determine the apparent Michaelis constant (Km) for NADH in the presence of varying concentrations of HAD. The Km of the reaction is inversely proportional to the Km for NADH, yielding the K/V ratio. For NADH and NADPH, K/V = 1.32 (not shown). Km (NADH) for 2-oxoglutarate was 2.8 mg, yielding K/V = 4.6 mg.

TABLE 2

| [H2O2] (mM) | Apparent K (mM) | Total H2O2 production (mM) | Extracellular H2O2 | [H2O2] (mM) |
|------------|----------------|---------------------------|-------------------|------------|
| Control    | 205 ± 3      | 100 ± 2       | 50 ± 2          | Control    |
| 0.05       | 190 ± 1      | 110 ± 2       | 40 ± 1          | 0.05       |
| 0.10       | 185 ± 1      | 105 ± 2       | 35 ± 1          | 0.10       |
| 0.25       | 150 ± 1      | 75 ± 2        | 75 ± 2          | 0.25       |
| 0.50       | 125 ± 1      | 225 ± 2       | 225 ± 2         | 0.50       |
| 1.00       | 100 ± 1      | 100 ± 1       | 100 ± 1         | 1.00       |
| 2.50       | 75 ± 1       | 75 ± 1        | 75 ± 1          | 2.50       |
| 5.0        | 50 ± 1       | 50 ± 1        | 50 ± 1          | 5.0        |

TABLE 3 - Effect of varying extracellular glucose and H2O2 concentrations on the rate of net NADH oxidation. (see Ch. 4) and the control strength of the transport step over the process of transport and phosphorylation of glucose (see Ch. 5). Values are mean ± S.D.

| Extracellular Glucose (mM) | H2O2 dose (mM) | Control Strength | Y2g (mMol/min/mg cells) |
|---------------------------|----------------|----------------|------------------------|
| 5000                      | 0              | 0.12           | 982                    |
| 100                       | 0              | 0.85           | 209 ± 27               |
| 100                       | 100            | 0.11           | 82 ± 6                 |
| 100                       | 0              | 0.92           | 18 ± 5.4               |
| 10                        | 100            | 0.90           | 16 ± 9.8               |

TABLE 4 - Apparent activity of the 2-oxoglutarate dehydrogenase, as assessed by formation of [3H]-2-oxoglutarate from [3H]-2-oxoglutarate and [3H]-glutamate. All values are mean ± S.D.

| [3H]-2-oxoglutarate (mM) | Control activity (mMol/min/mg cells) | Stimulated increase (mMol/min/mg cells) | Glucose metabolism through TCA cycle (mMol/min/mg cells) | Stimulated activity (mMol/min/mg cells) | number of experiments |
|--------------------------|-------------------------------------|----------------------------------------|----------------------------------------------------------|----------------------------------------|----------------------|
| Control                  | 124 ± 6                            | 1.2 ± 0.9                              | 1.2 ± 0.9                                                | 1.2 ± 0.9                              | 16                   |
| 0.05                     | 160 ± 8                            | 1.3 ± 0.9                              | 1.3 ± 0.9                                                | 1.3 ± 0.9                              | 2                    |
| 0.10                     | 180 ± 10                           | 1.6 ± 1.0                              | 1.6 ± 1.0                                                | 1.6 ± 1.0                              | 3                    |
| 0.25                     | 200 ± 5                            | 1.7 ± 2.0                              | 1.7 ± 2.0                                                | 1.7 ± 2.0                              | 4                    |
| 0.50                     | 250 ± 1                            | 1.8 ± 2.0                              | 1.8 ± 2.0                                                | 1.8 ± 2.0                              | 3                    |
| 1.00                     | 300 ± 1                            | 1.9 ± 2.0                              | 1.9 ± 2.0                                                | 1.9 ± 2.0                              | 3                    |
| 2.50                     | 400 ± 1                            | 2.0 ± 2.0                              | 2.0 ± 2.0                                                | 2.0 ± 2.0                              | 3                    |
| 5.0                      | 500 ± 1                            | 2.1 ± 2.0                              | 2.1 ± 2.0                                                | 2.1 ± 2.0                              | 3                    |

TABLE 5 - Kinetic parameters of P3823, glycogen phosphorylase. Activities were determined in four separate experiments. Michaelis constants were determined from curves from 100 substrates. Values obtained from curve. Kinetic parameters of P3823, glycogen phosphorylase. Values obtained from curve.
Mechanisms of Oxidant Injury

TABLE 5

| H2O2 | G-6-P (mol/mg cell) | F-6-P (mol/mg cell) | GAP (mol/mg cell) | GAP + [3H] (mol/mg cell) |
|------|---------------------|---------------------|-------------------|-------------------------|
| Control | 1.40 ± 0.05 | 0.39 ± 0.03 | 0.19 ± 0.03 | 0.19 ± 0.03 |
| 0.05 | 0.66 ± 0.02 | 0.17 ± 0.12 | 0.12 ± 0.06 | 0.12 ± 0.06 |
| 0.10 | 0.64 ± 0.05 | 0.20 ± 0.05 | 0.18 ± 0.05 | 0.18 ± 0.05 |
| 0.25 | 0.66 ± 0.02 | 0.16 ± 0.02 | 0.19 ± 0.05 | 0.19 ± 0.05 |
| 0.50 | 0.30 ± 0.02 | 0.16 ± 0.02 | 0.11 ± 0.01 | 0.11 ± 0.01 |
| 1.00 | 0.30 ± 0.02 | 0.12 ± 0.03 | 0.09 ± 0.03 | 0.09 ± 0.03 |
| 2.50 | 0.26 ± 0.07 | 0.07 ± 0.03 | 0.05 ± 0.02 | 0.05 ± 0.02 |
| 5.00 | 0.25 ± 0.08 | 0.06 ± 0.06 | 0.03 ± 0.02 | 0.03 ± 0.02 |

TABLE 6

Table 6 - The effect of NADH concentration on cell survivability following a 15 min incubation, and transfer to full growth media. Plots of log cell number as a function of time in the growth media yielded doubling times (column 3) and N0, the number of cells potentially surviving following H2O2 injury.

| Time after H2O2 addition (min.) | Estimated Cytosolic [NADH] (µM) | Estimated Cytosolic [NAD] (µM) | Theoretical rate of NAD oxidation (nmol/min.) |
|---------------------------------|----------------------------------|---------------------------------|-----------------------------------------------|
| 0                               | 152.6                            | 12.6                            | 50.6                                          |
| 5                               | 49.2                             | 24.4                            | 45.4                                          |
| 10                              | 19.6                             | 12.7                            | 23.3                                          |
| 15                              | 14.2                             | 11.6                            | 28.8                                          |
| 20                              | 13.5                             | 10.8                            | 26.8                                          |
| 24                              | 11.8                             | 9.6                             | 26.0                                          |
| 30                              | 9.3                              | 7.4                             | 21.6                                          |

TABLE 7

Table 7 - The effect of NADH on cell survivability following a 15 min incubation, and transfer to full growth media. Plots of log cell number as a function of time in the growth media yielded doubling times (column 3) and N0, the number of cells potentially surviving following H2O2 injury.

| (H2O2) (nM) | NADH (µM) | Doubling time (h) | Correlation coefficient |
|-------------|-----------|------------------|------------------------|
| 0.00        | 4.14 x 10^5 | 1.4 | 0.597 |
| 0.05        | 5.44 x 10^5 | 12.0 | 0.940 |
| 0.10        | 4.73 x 10^5 | 11.9 | 0.986 |
| 0.20        | 4.26 x 10^5 | 12.9 | 0.980 |
| 0.30        | 3.29 x 10^5 | 11.8 | 0.999 |
| 0.40        | 3.16 x 10^5 | 12.0 | 0.999 |
| 0.50        | 3.07 x 10^5 | 12.0 | 0.999 |
| 0.60        | 2.76 x 10^5 | 12.0 | 0.999 |
| 0.70        | 2.15 x 10^5 | 12.6 | 0.996 |
| 0.80        | 1.12 x 10^5 | 12.4 | 0.994 |

FIGURE 1 - Estimation of the control strength of the hexose transport step at 100 µM extracellular glucose, utilizing tritiated glucose as tracer, and cytochalasin B as a non-competitive inhibitor of hexose transport. Control strength values were interpreted from the tangents to the initial segments of the curves (see text). Upper panel: tracer uptake with and without cytochalasin B. Lower panel: tracer uptake in control cells.

FIGURE 2 - Solid circles: Inhibition of lactate dehydrogenase by 15 µM 2,4-DNPH exposure to 10,000 cells. LDH activity was assessed in the presence and absence of partially purified L-lactate dehydrogenase. Open circles: estimation of purified lactate dehydrogenase activity in the presence and absence of partially purified NADH dehydrogenase. 50% of OD, 100% of OD. The enzyme was inhibited and assayed in the same manner as the HAD.
Mechanisms of Oxidant Injury

**FIGURE 1** - Time-course of the effects of H$_2$O$_2$ on the intracellular content of NADH (panel A) and NAD$^+$ (panel B) in P388D cells. The data is presented as mean ± SEM from duplicate determinations in 200, 500 and 1000 μM H$_2$O$_2$. Panel C: Time course of inhibition of P388D intracellular NAD$^+$ activity by 200 and 500 μM H$_2$O$_2$. Panel D: Time course of inhibition of hexokinase sensitive ATPase in P388D cells exposed to 500 μM H$_2$O$_2$.

**FIGURE 2** - Binding of $^3$H NADH to reconstituted P388D cytosol (post 100,000 x g supernatant) material. The supernatant was reconstituted to the original lactate dehydrogenase specific activity, and free ($^3$H) binding ratio of increasing nucleotide concentrations determined as in the text. Filled squares: NAD$, open squares, NADH. Error bars represent upper or lower limits of the S.D. (n = 3).
Mechanisms of Oxidant Injury

![Graph A](image1)

**FIGURE 1** - Left panel: Oxygen consumption of P388D cells as the difference between basal (open circles) or respiration inhibited by the addition of (open squares) antimycin A. Right panel: Oxygen consumption (closed circles) as a function of H$_2$O$_2$ exposure. Uncoupling was induced by the addition of CCCP. Dashed lines represent the upper or lower limits of the standard deviation of 5 separate experiments. All oxygen consumption rates were cyanide suppressible.

![Graph B](image2)

**FIGURE 2** - Intracellular ATP content of P388D cells as a function of time. (a) control; (b) cells exposed to antimycin A at time zero; (c) 2-deoxy-D-glucose (10 mM); and (d) 2-deoxy-D-glucose plus 2-deoxy-D-glucose. The approximate steady-state rate of intracellular ATP hydrolysis was estimated from the dashed line (2.1 nmol ATP/10$^6$ cells).

![Graph C](image3)

**FIGURE 3** - Intracellular ATP determinations as a function of H$_2$O$_2$ exposure for 15 min. Values are means ± S.D. (n = 4). Values within brackets labelled in the figure are H$_2$O$_2$ concentrations in micro molar.