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Protective Effect of Glutamine on Endothelial Cell ATP in Oxidant Injury[1]

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Endothelial cell dysfunction following exposure to H_2O_2 is associated with rapid inhibition of glucose-dependent pathways of ATP synthesis. The role other substrates for ATP synthesis (e.g., amino acids) may play in the metabolism of H_2O_2-injured cells is unclear. The effect of glutamine, a precursor of the Kreb's cycle intermediate α-ketoglutarate on ATP levels in bovine pulmonary artery endothelial cells exposed to H_2O_2 was examined. The presence of glutamine during H_2O_2 injury significantly enhanced ATP levels in the injured cells. Concentrations of glutamine as low as 50 μM produced significant improvement of ATP levels in endothelial cells exposed to 5 mM H_2O_2. The 2 mM concentration of glutamine produced the greatest benefit, while greater concentrations of glutamine (5–20 mM) were actually associated with progressive decrements of the maximal benefit seen with the 2 mM concentration. The 2 mM concentration of glutamine produced similar enhancement of ATP with 1 and 10 mM H_2O_2 injury as well. Short-term viability following 5 mM H_2O_2 injury was significantly improved by the presence of 2 mM glutamine. The most effective concentration of glutamine (2 mM) did not scavenge H_2O_2 in a fluorometric assay. These observations suggest that mitochondrial substrates, such as glutamine, that bypass glucose-dependent pathways of ATP synthesis may be useful therapeutic agents for maintenance of ATP levels in oxidant-injured cells.

INTRODUCTION

Hydrogen peroxide (H_2O_2) has been implicated as one of the major oxidants responsible for cellular injury mediated by polymorphonuclear leukocytes (PMN) during inflammation [1–4]. A number of biochemical events occur in injured cells following exposure to H_2O_2. These include DNA strand breakage with activation of the nuclear enzyme poly-(ADP-ribose) polymerase [5, 6]; activation of the glutathione (GSH) redox cycle with oxidation or loss of GSH [7, 8]; loss of intracellular Ca^{2+} homeostasis [8, 9]; and perhaps most important for short-term cellular survival and function, inhibition of ATP synthesis [10, 11]. Reduction of cellular ATP levels in oxidant injury is followed by disruption of microfilaments, important structural determinants of the cell [8, 12–14].

The key glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is inhibited by H_2O_2 and this inhibition accounts for the loss of aerobic and anaerobic glycolytic activity in H_2O_2-injured cells [11, 15, 16]. Oxidative phosphorylation of ADP in mitochondria of H_2O_2-injured cells is also inhibited but appears to be somewhat less sensitive than glycolysis [11]. In fact, studies with the P388D$_1$ cell line suggest that at intermediate concentrations of H_2O_2, ATP synthesis by mitochondrial pathways may be accelerated in response to the H_2O_2-mediated inhibition of glycolysis [11].

Although glucose-dependent pathways (aerobic and anaerobic glycolysis) may be inhibited by oxidant injury, it is possible that other substrates, particularly those metabolized via the Kreb's cycle, could serve as sources of energy in cells injured by H_2O_2. Branched chain amino acids and glutamine have been demonstrated to be oxidizable sources of energy in isolated perfused organs [17, 18]. *In vitro* studies have revealed a high level of glutaminase activity in endothelial cells [19]. Glutamine may be metabolized primarily for biosynthetic purposes or catabolized for the generation of ATP depending on the cell and the availability of other substrates [20–22].

It is unclear whether other potentially oxidizable substrates such as glutamine can be used for ATP synthesis in cells injured by oxidants. The purpose of this study was to examine this possibility in a model of H_2O_2 injury to endothelial cells.

METHODS

Cells and Culture

Bovine pulmonary artery endothelial cells (No. AG 2791A) from the National Institute of Aging, Aging Cell

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Culture Repository (Camden, NJ) were grown to confluence in 150-cm^2 flasks (Corning). Their culture media included RPMI 1640 containing 2 m*M* glutamine supplemented with 10% fetal bovine serum (Whittaker, M. A. Bioproducts), 10 m*M* Hepes, 100 u/ml penicillin, and 100 μg/ml streptomycin (GIBCO). Cells were passaged after a brief exposure (10–15 min) to trypsin (0.05%) and EDTA (0.02%; Sigma). Cells from the fifth to the seventh passages were used for these experiments. The cells were suspended at a concentration of 2×10^6/ml in modified Gey's buffer (MGB) containing 147 m*M* NaCl, 5 m*M* KCl, 1.9 m*M* KH_2PO_4, 1.1 m*M* Na_2HPO_4, 0.3 m*M* $MgSO_4$, 1 m*M* $MgCl_2$, 5.5 m*M* glucose, 10 m*M* Hepes, and 1.5 m*M* $CaCl_2$. In some experiments, glutamine (0–20 m*M*) was present in the buffer.

Experiments with suspended cells were carried out at 37°C in a shaking water bath.

Cell Viability Measurements

Cellular viability was estimated at different time points by the ability of the cells to exclude trypan blue [9]. The cells (50 μl of 2×10^6 cells/ml) were incubated briefly with 12 μl of 0.1% trypan blue in 0.9% NaCl (final concentration approx 0.02%) and the number of cells excluding trypan blue out of 100 cells counted on a hemacytometer was equal to the percentage viable cells.

ATP Determinations

Endothelial cell ATP levels were assayed by the modified luciferase–luciferin method of Stanley and Williams [13, 23]. The luciferase–luciferin (Sigma No. LO633) was reconstituted in a buffer containing 1% bovine serum albumin, 20 m*M* glycine, and 2 m*M* EDTA, pH 8.0. Measurements were performed in a LKB Model 1251 automated luminometer. ATP data are expressed as nanomoles (10^{-9} mole) ATP per 2×10^6 cells.

H_2O_2 Measurements

Measurement of a range of H_2O_2 concentrations in the presence or absence of glutamine was done using a fluorometric assay [24]. The assay is based on the conversion of nonfluorescent *para*-hydroxyphenyl acetic acid (PHPA) to a fluorescent dimer $(PHPA)_2$ by reaction with H_2O_2 in the presence of the enzyme horseradish peroxidase. A cocktail of 166.5 μg/ml PHPA (Sigma) and 53.4 μg/ml horseradish peroxidase (Sigma) was added to 2 ml of stirred MGB. Small 5- to 10-μl aliquots taken from a 2 m*M* stock solution of H_2O_2 (Fisher) were added and the fluorescence was read in a SLM 8000 C spectrofluorometer at 37°C with excitation at 323 nm and emission at 400 nm. H_2O_2 final concentrations of 0–70 μM produced near linear increases of $(PHPA)_2$ fluorescence under these conditions.

Statistical Analysis

Two-way analysis of variance was used for the statistical evaluation of the data unless otherwise indicated.

RESULTS

Endothelial Cell ATP and H_2O_2 Injury—Glutamine Dose Response

Earlier work with the bovine pulmonary artery endothelial cells [8] demonstrated that injury with a bolus of 5 m*M* H_2O_2 produced significant reduction of endothelial ATP levels and elevation of intracellular Ca^{2+}. Since this was the lowest dose of H_2O_2 tested which affected both parameters of endothelial injury, it was chosen to study the effect of a range of glutamine concentrations (0–20 m*M*) on ATP synthesis during H_2O_2 injury. The endothelial cells were incubated in MGB containing 5.5 m*M* glucose and the different concentrations of glutamine for 20 min at 37°C prior to addition of H_2O_2 (final concentration, 5 m*M*). Figure 1 depicts the dose response of ATP levels in H_2O_2-injured endothelial cells to treatment with glutamine. Significant elevation ($P < 0.05$, *t* test) of ATP levels in the injured cells treated with glutamine was seen as early as 30 min after addition of H_2O_2 with concentrations of glutamine ≥ 500 μM. Cell counts done over the time course demonstrated that cell numbers remained constant during the injury. This indicated that reduction of ATP in the injured samples was not due to a loss of cells as a result of the injury. The lowest concentration of glutamine that produced significant dose- and time-dependent enhancement of ATP levels in cells injured with 5 m*M* H_2O_2 over the 3-hr time course was 50 μM ($P < 0.05$). The optimal effect was achieved with 1–2 m*M* glutamine ($P < 0.0001$). Concentrations of glutamine > 2 m*M* were associated with a decreasing beneficial effect on ATP levels. Indeed, there was no significant benefit seen with glutamine concentrations > 5 m*M*.

Glutamine Effects on Endothelial Cell ATP and Viability—H_2O_2 Dose Response

ATP levels were examined in endothelial cells treated with 2 m*M* glutamine prior to and during injury with 0–10 m*M* H_2O_2 (Fig. 2). After 30 min of injury glutamine maintained ATP in H_2O_2-injured cells at a level severalfold higher than the level induced by 5 or 10 m*M* H_2O_2 in the absence of glutamine. The effect of glutamine on ATP levels after 1 m*M* H_2O_2 injury was less dramatic but 2 m*M* glutamine had a significant effect on ATP levels over the 3-hr time course at all the H_2O_2 concentrations tested ($P < 0.05$). Cells treated with glutamine and exposed to the different concentrations of H_2O_2 still had ATP levels significantly lower than those of uninjured control cells ($P < 0.0001$). Glutamine supplementation

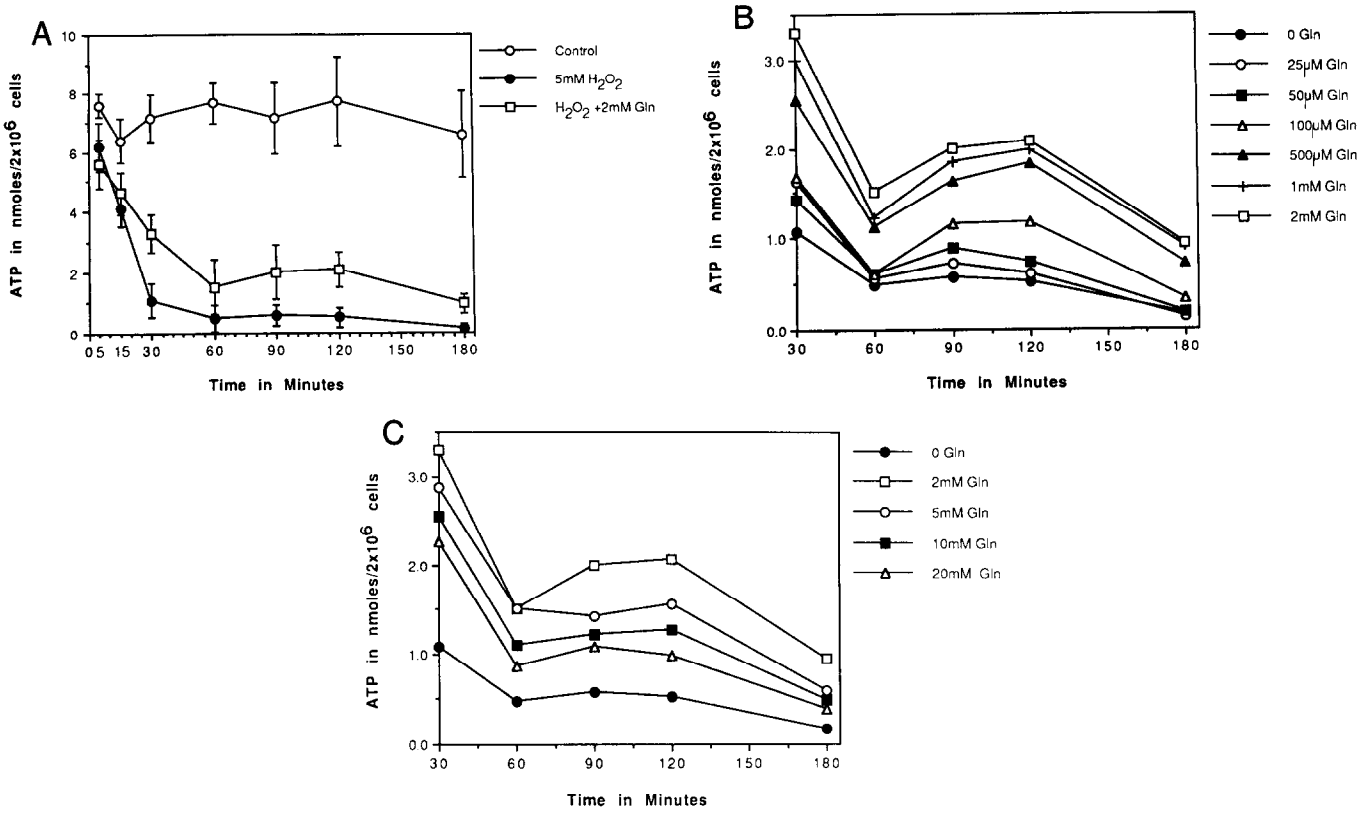

FIG. 1. The effect of glutamine (0–20 m*M*) on ATP levels in endothelial cells exposed to 5 m*M* H_2O_2. (A) Full time course with 2 m*M* glutamine. Data points represent the $x \pm$ SD of four to six separate determinations. (B) Time course from 30–180 min after H_2O_2 addition with 0–2 m*M* glutamine present (expanded scale). SD error bars have been removed for clarity. (C) Time course from 30 to 180 min after H_2O_2 addition with 2–20 m*M* glutamine present (expanded scale). SD error bars have been removed for clarity.

alone without injury did not significantly enhance ATP levels in the control cells.

The parallel effect of glutamine on viability was also examined in the same experiments. Figure 3 depicts the 5-hr time course of short-term viability seen in H_2O_2-injured cells treated with glutamine. Glutamine-mediated effects on trypan blue exclusion in H_2O_2-injured cells became apparent later than differences in ATP levels associated with glutamine treatment. Glutamine did significantly enhance viability in uninjured control cells ($P < 0.05$). Cells injured with 5 m*M* H_2O_2 and treated with 2 m*M* glutamine exhibited significant improvement of

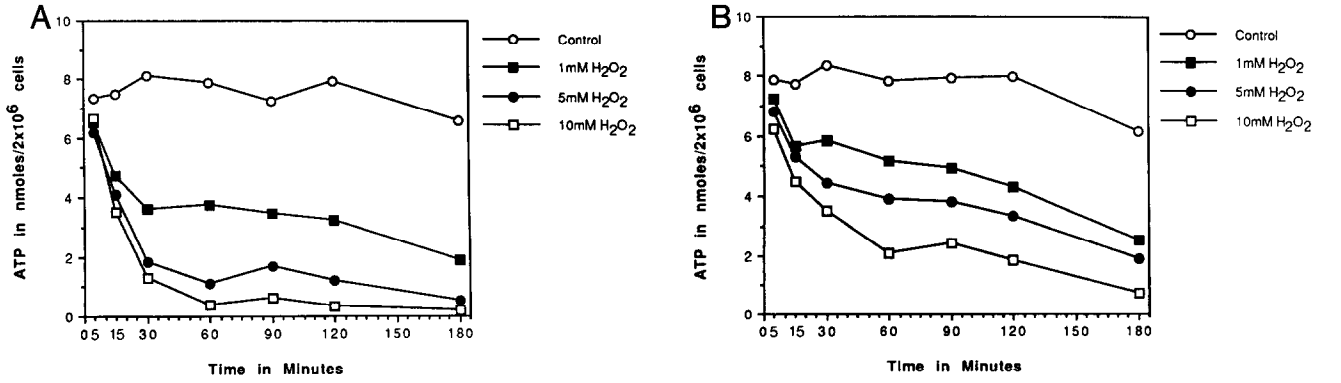

FIG. 2. The effect of 2 m*M* glutamine on ATP levels in endothelial cells exposed to 0–10 m*M* H_2O_2. (A) Time course of endothelial ATP levels after addition of 0–10 m*M* H_2O_2. (B) Time course of endothelial ATP levels after addition of 0–10 m*M* H_2O_2 in the presence of 2 m*M* glutamine. Data points represent the x of four to six separate determinations. SD error bars have been deleted for clarity.

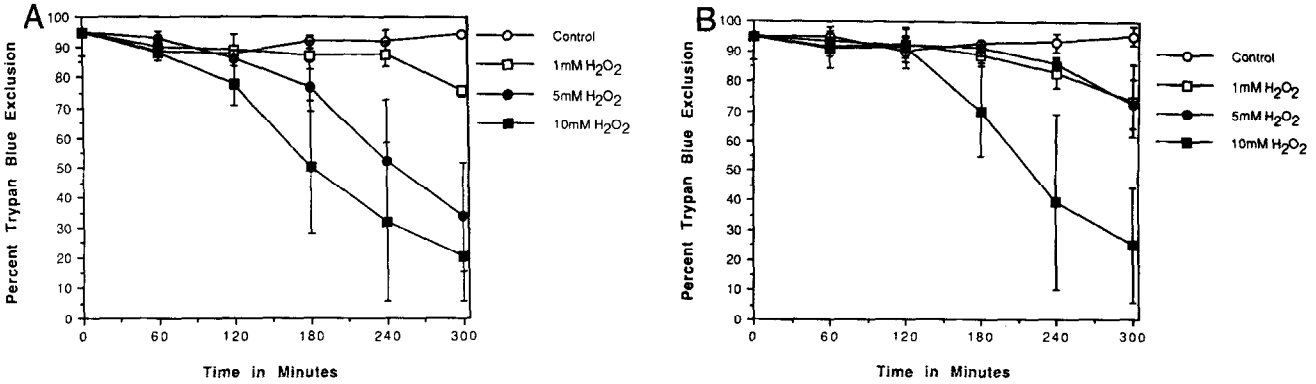

FIG. 3. The effect of 2 m*M* glutamine on short-term viability in endothelial cells exposed to 0–10 m*M* H_2O_2. (A) Time course of endothelial viability (% trypan blue exclusion) following exposure to 0–10 m*M* H_2O_2. (B) Time course of endothelial viability (% trypan blue exclusion) following exposure to 0–10 m*M* H_2O_2 in the presence of 2 m*M* glutamine. Data represent the $x \pm$ SD of three separate determinations.

short-term viability ($P < 0.0001$). Glutamine did not significantly improve short-term viability in cells injured with 1 or 10 m*M* H_2O_2.

Glutamine and H_2O_2

One possible explanation for the beneficial effect of glutamine on ATP levels and viability in oxidant-injured endothelial cells could be that it may act as a scavenger of H_2O_2. To test this hypothesis, standard curves of $(PHPA)_2$ fluorescence (which is directly proportional to H_2O_2 concentration) were constructed in the presence of 0–20 m*M* glutamine. Glutamine had no significant effect on the reaction of H_2O_2 with the fluorometric assay reagents (Fig. 4), except at the 20 m*M* concentration ($P < 0.001$). The highest concentration of glutamine did blunt the standard curve of $(PHPA)_2$ fluorescence in response to H_2O_2.

DISCUSSION

The presence of glutamine during H_2O_2 injury was associated with significantly enhanced endothelial cell ATP levels and short-term viability (at the intermediate concentration of H_2O_2). The optimal concentration of glutamine was approx 2 m*M*. It is unclear why the higher concentrations of glutamine were associated with no additional benefit and in fact with an apparent adverse effect on ATP levels. A 10-fold increase in glutamine (20 m*M*) did not alter the pH of the experimental buffer (data not shown). This tends to mitigate against the possibility that there is a substantially greater NH_3 load from the higher glutamine concentrations. It is possible, though, that the additional NH_3 load may exert its primary effect on intracellular pH. This will require future experimental confirmation, however.

Glutamine significantly enhanced ATP levels in injured cells at all the concentrations of H_2O_2 tested. However, it did not restore ATP in the injured cells to the control range. Short-term viability measured by exclusion of trypan blue was improved to a much greater degree following injury with 5 m*M* H_2O_2 than with either 1 or 10 m*M* H_2O_2. This is interesting in light of the effect glutamine had on ATP levels at these concentrations of H_2O_2. It is possible that significant differences in viability of glutamine-treated cells exposed to 1 m*M* H_2O_2 may become manifest at later time points. Under these experimental conditions it appears that enhancing ATP levels alone may not necessarily enhance short-term viability (e.g., 10 m*M* H_2O_2 data). From unpublished work in our laboratory it appears that a degree of enhanced short-term viability similar to that seen with cells injured by 5 m*M* H_2O_2 and treated with 2 m*M* glutamine can be achieved following exposure to 10 m*M* H_2O_2 if the cells have a media change the day prior to the experiment. The culture media contains 2 m*M* glutamine. A limitation of studies of this kind is that the suspended cells have a gradual decline in ATP levels when kept in simple buffers like MGB that contain glucose as the primary substrate. To separate short-term

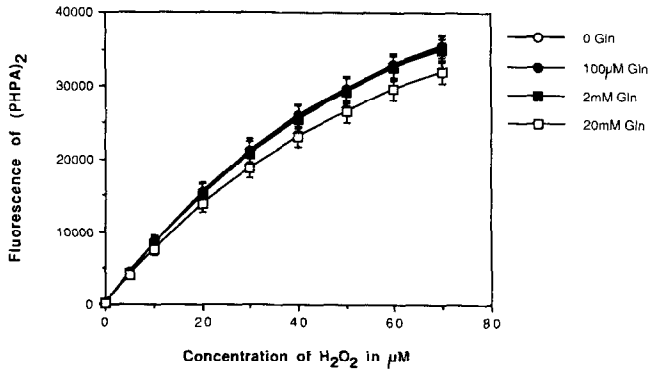

FIG. 4. The effect of glutamine on measurement of H_2O_2 by a fluorometric assay. Data represent the $x \pm$ SD of four separate determinations.

effects of injury from experimental conditions such as the constituents of the buffer, it will be necessary to repeat these observations with cells kept sterile during injury for later culture so that viability, cell number, ATP levels, etc., may be determined 24–72 hr or longer after growth in culture. It is interesting to note that viability of the control cells over 5 hr in the simple buffer was significantly enhanced by the presence of 2 m*M* glutamine, although ATP levels were not affected. This observation is consistent with other observations [20, 22] regarding glutamine's potential role as a substrate for biosynthetic activities in cells.

The fluorometric assay for H_2O_2 supports the concept that glutamine did not enhance ATP levels and cellular viability by acting as a scavenger of H_2O_2. The concentration associated with the greatest benefit, 2 m*M*, had no effect on the measurement of H_2O_2 in the fluorometric assay. Although 20 m*M* glutamine did affect the measurements, this was a concentration associated with a substantially decreased beneficial effect on ATP levels compared to 2 m*M* glutamine. Indeed, 20 m*M* glutamine may interfere with the assay rather than actually scavenge H_2O_2. The data do support a role for glutamine as an alternative substrate for ATP synthesis in oxidant-injured cells.

An unanswered question from this data is the long-term effect of glutamine on cellular viability and function. It is quite possible that enhancement of ATP levels in injured cells may only delay the inevitable, particularly if failure of ATP synthesis is not the *sine qua non* of lethal cell injury.

H_2O_2-mediated inhibition of glycolysis by oxidative inactivation of GAPDH occurs rapidly [11]. The data presented here may be interpreted to suggest that mitochondrial ATP synthesis is inhibited more slowly by oxidants than the glycolytic pathway. This would be consistent with gradual loss of overall mitochondrial integrity by diffuse processes (e.g., altered mitochondrial Ca^{2+} homeostasis) in contrast to specific inhibition of an enzyme(s) in the Kreb's cycle. Glutamine may be a useful tool for further elucidation of the mechanism of injury to mitochondria mediated by oxidants.

A major implication of the effect of glutamine on metabolism in oxidant-injured cells may be in relation to the known loss of glutamine from skeletal muscle following trauma and sepsis [25]. Oxidant-injured cells present in the tissues of a traumatized or septic patient may constitute a potential "sink" for glutamine and help to further drive the protein catabolism in skeletal muscle.

In summary, the presence of glutamine significantly enhanced ATP levels and short-term viability of endothelial cells exposed to H_2O_2. These observations may provide new insights into the mechanism of cellular injury mediated by oxidants and may also have therapeutic implications.

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