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Biphasic ATP Depletion Caused by Transient Oxidative Exposure Is Associated with Apoptotic Cell Death in Rat Embryonal Cortical Neurons

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ABSTRACT

Hypoxia-ischemia leads to an acute depletion of high-energy phosphates in neonatal brain. After reperfusion, energy status is restored, but may show progressive secondary failure, associated with neuronal loss, brain damage, or death. Oxidants are produced on reperfusion. We investigated whether a biphasic energy failure develops in cultured neurons after oxidant exposure, and whether the degree of primary disturbance correlates with later ATP synthesis and mode of cell death. Embryonic rat cortical neurons were exposed to varying doses of hydrogen peroxide for 60 min and incubated for 12, 24, or 48 h. Adenine nucleotides and the incorporation of [^{14}C]adenine into adenine nucleotides were quantified. Apoptosis was evaluated by DNA electrophoresis and *in situ* end-labeling. A mild insult (10–50 μM) caused no ATP depletion or change in subsequent growth or energy metabolism, whereas an intermediate insult (100 μM) caused acute ATP depletion (49 ± 12% of control). This recovered to 91 ± 28% by 12 h, but then declined to 61 ± 18% at 24 h. A severe insult (1 mM) depleted ATP to 15 ± 3% of control, with no recovery. Moderate ATP depletion was associated with apoptotic cell death, whereas a severe insult caused acute necrosis. Transient oxidant exposure of embryonal cortical neurons causes a biphasic energy depletion followed by apoptosis in analogy with asphyxiated brains. This model may prove useful for the study of pathogenesis and treatment of hypoxic-ischemic encephalopathy. **(*Pediatr Res* 52: 40–45, 2002)**

Abbreviations

ANs, adenine nucleotides
DIG-ddUTP, digoxigenin-11-ddUTP
EC, energy charge
H_2O_2, hydrogen peroxide
ISEL, *in situ* end-labeling
PARP, poly(ADP ribose) polymerase
PCr, phosphocreatine
P_i, inorganic phosphate
TdT, terminal transferase

Hypoxia-ischemia in a neonatal piglet model leads to an acute decrease of high-energy phosphates in the brain, reflected in a decrease of the ratio of PCr to P_i (PCr/P_i) measured by ^{31}P-nuclear magnetic resonance spectroscopy. After reperfusion, the energy status is initially restored, but in severe cases, a progressive secondary failure may develop, starting some 12 h later and reaching its nadir at 48 h (1, 2). Human infants cannot be studied at the time of asphyxia, but after resuscitation the PCr/P_i of the brain is in the normal range. In severe cases, after the first day of life, a delayed impairment develops. The degree of this presumably secondary energy failure correlates with clinical outcome (3–5). A biphasic cerebral energy failure after asphyxia is reproducible also in a rat model and in fetal sheep (6, 7). The degree of high-energy phosphate depletion during the primary insult correlates with the number of apoptotic and necrotic cells in the brains of asphyxiated piglets at 48 h (8), and with the degree of cerebral infarction in developing rats (6). In an immature rat model, the neuronal death caused by a short ischemic period may mostly be accounted for by apoptosis, whereas severe ischemia may produce immediate or delayed necrosis (9).

Acute disruption of oxidative phosphorylation during ischemia leads to loss of ion homeostasis, particularly a massive calcium influx, which has multiple effects on cellular functions. If long-lasting enough, ischemia produces immediate cell death. After a period of nonfatal cerebral ischemia, reperfusion is associated with increased mitochondrial or cytoplasmic generation of reactive oxygen metabolites, *e.g.* superoxide, which is converted to H_2O_2 by superoxide dismutases [reviewed by Fellman and Raivio (10)]. H_2O_2 is a freely diffusing oxidant, which *in vitro* triggers neuronal apoptosis (11), leading to acute and delayed ATP depletion (12).

Although it is known that in the absence of cellular ATP the apoptotic program can switch to necrosis (13–16), the correlation of the acute ATP depletion caused by an oxidative insult

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with later energy metabolism in cortical neurons has not been investigated. Therefore, the aim of this study was to test whether the initial disturbance in the balance of high-energy nucleotides caused by a transient oxidative insult is reversible and whether its severity would predict later ATP synthesis and subsequent mode of cell death *in vitro*. A biphasic disturbance in energy metabolism would correlate with that observed in the brains of asphyxiated human infants and neonatal animals, providing a reproducible model for studying some components of hypoxic-ischemic neuronal injury.

METHODS

Cell culture. Cells from cerebral hemispheres of 18-d-old rat embryos were prepared as previously described (17). Freshly prepared neurons in Dulbecco's modified Eagle's medium (DMEM, GIBCO Europe, Paisley, U. K.), supplemented with 10% fetal bovine serum and containing penicillin–streptomycin (100 IU/mL and 50 μg/mL), were plated on poly-L-lysine–coated four-well culture dishes (Nunc, Roskilde, Denmark) or 50-mm Petri dishes (Nunc) at a controlled density averaging 750 cells/mm^2 and incubated in 95% air/5% CO_2 at 37°C in a humidified incubator. The next day the culture medium was changed to serum-free Neurobasal medium supplemented with B27 (GIBCO), and replaced after 4 d. These cells have been previously characterized and shown to be mainly (80–90%) neurons (17). This primary cell culture system has been approved by the Ethical Board of the University of Helsinki.

Oxidant exposure. On d 5–7, when the cells were showing marked neuronal outgrowth, the medium was replaced with serum-free DMEM containing freshly prepared H_2O_2 at a final concentration of 0 μM, 10 μM, 50 μM, 100 μM, 1 mM, or 5 mM (Merck AG, Darmstadt, Germany) and incubated at 37°C for 60 min. The H_2O_2 concentrations were verified by spectrophotometric analysis at 240 nm, using the molar extinction coefficient of 44 $M^{-1}\cdot cm^{-1}$ for H_2O_2. Control cells were treated equally but with omission of H_2O_2.

After exposure, the cells were either collected immediately for analysis or incubated further in defined culture medium (Neurobasal + B27) for 12, 24, or 48 h, and then analyzed. For DNA analysis, the experiments were performed on Petri dishes.

Analysis of DNA degradation. The exposed cells as well as their unexposed controls were scraped into medium, pelleted, resuspended in 200 μL of PBS, and quickly frozen in liquid nitrogen. DNA was extracted using the Apoptotic DNA ladder kit (Roche Biochemicals, Basel, Switzerland), quantified, and electrophoresed (3 μg/lane, 50 V, 3.5 h) on 2% standard low-melting agarose gel, then visualized with a fluorescent DNA-binding dye SYBR Gold (Molecular Probes Europe, Leiden, The Netherlands) and photographed under UV illumination.

ISEL of DNA. The neurons were plated on poly-L-lysine–coated round glass coverslips on four-well culture dishes (Nunc) and grown as described earlier. They were then exposed to indicated concentrations of H_2O_2 for 60 min, incubated in defined culture medium for 24 h, washed twice, and fixed in 4% paraformaldehyde. The cells were permeabilized in ice-cold mixture of ethanol and acetic acid (2:1), and after washing, placed in a microwave oven for 5 min in 10 mM citric acid (pH 6.0). After washing, the cells were preincubated with terminal transferase reaction buffer (potassium cacodylate, 1 M; Tris-HCl, 125 mM; BSA, 1.25 mg/mL; pH 6.6). The apoptotic DNA fragments were 3'-end-labeled with DIG-ddUTP (Roche Diagnostics, Mannheim, Germany) by the TdT (Roche Diagnostics) reaction for 1 h at 37°C. Antidigoxigenin antibody conjugated to horseradish peroxidase (1:50, Roche Diagnostics) and diaminobenzidine (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used to detect DIG-ddUTP-labeled DNA. For the negative controls, the TdT enzyme was replaced by the same volume of distilled water. The coverslips were then dehydrated, mounted, and photographed under a light microscope (×40, interference contrast).

AN depletion assay. Before the exposure, cells were prelabeled overnight with [^{14}C]adenine (final concentration, 100 μM; specific activity, 287 mCi/mmol; Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, Buckinghamshire, U.K.) in defined culture medium. The labeled medium was then removed, and cells were exposed as appropriate. Directly after exposure, the medium was removed, and the cellular ANs were extracted on ice with perchloric acid. ATP, ADP, and AMP were separated by thin-layer chromatography, and the radioactivities in the ANs, which correspond to their intracellular concentrations, were measured with a liquid scintillation counter (Rackbeta 1209, LKB Wallac, Turku, Finland) (18). The adenylate EC, defined as [([ATP] + 1/2 [ADP])/([ATP] + [ADP] + [AMP])], was used as a measure of cellular metabolic integrity (19).

Labeling of the AN pool. To measure the incorporation of [^{14}C]adenine into cellular nucleotides after an oxidant exposure, the culture medium of exposed as well as unexposed control cultures was supplemented with [^{14}C]adenine (final concentration 100 μM). After further incubation for 12 or 24 h, the cellular ANs were extracted with perchloric acid on ice and quantified (18).

Analysis of ANs by HPLC. ATP, ADP, and AMP concentrations in the neutralized perchloric acid extracts were measured using a Shimadzu LC 10AD vp liquid chromatograph with a reversed-phase column (Ultra Techsphere 5 ODS, Labtronic Oy, Vantaa, Finland) and a UV detector set at 254 nm. The published method (20) was modified as follows: buffer A (0.1 M KH_2PO_4, 8.0 mM tetrabutylammonium hydrogen sulfate, pH 6.0) was run at 1.5 mL/min for 2.5 min followed by a linear increase for 10 min to 100% buffer B (buffer A with 30% methanol), which was continued for 5 min. Compounds were identified and quantified by the retention times and peak areas of known standards, calibrated by spectrophotometry.

Protein analysis. Cells from parallel cocultures were homogenized in potassium phosphate buffer, and their protein content was determined by spectrophotometry using the Bio-Rad DC kit (Bio-Rad, Hercules, CA, U.S.A.).

Data analysis. Results are expressed as mean ± SD of two to four independent experiments performed at least in duplicate, each with its own control. The statistical group comparisons were made among control and test conditions by ANOVA combined with Bonferroni *post hoc* test (SPSS 10.0

Software for Windows, SPSS, Chicago, IL, U.S.A.). A $p < 0.05$ was considered significant. For illustration, the data are also shown as percent of control.

RESULTS

Cell and DNA damage. When examined after 24 h, the cells exposed for 60 min to 10–50 μM of H_2O_2 did not differ morphologically from control cells showing intact neurites and only light staining by ISEL (Fig. 1*A*), whereas cells exposed to 100 μM H_2O_2 were round and pyknotic and showed uniform ISEL (Fig. 1*B*). Cells exposed to a higher concentration of H_2O_2 (1 mM) formed a necrotic mass with distorted cell outlines at 24 h, but ISEL showed a scanty pattern of nuclear staining (Fig. 1*C*).

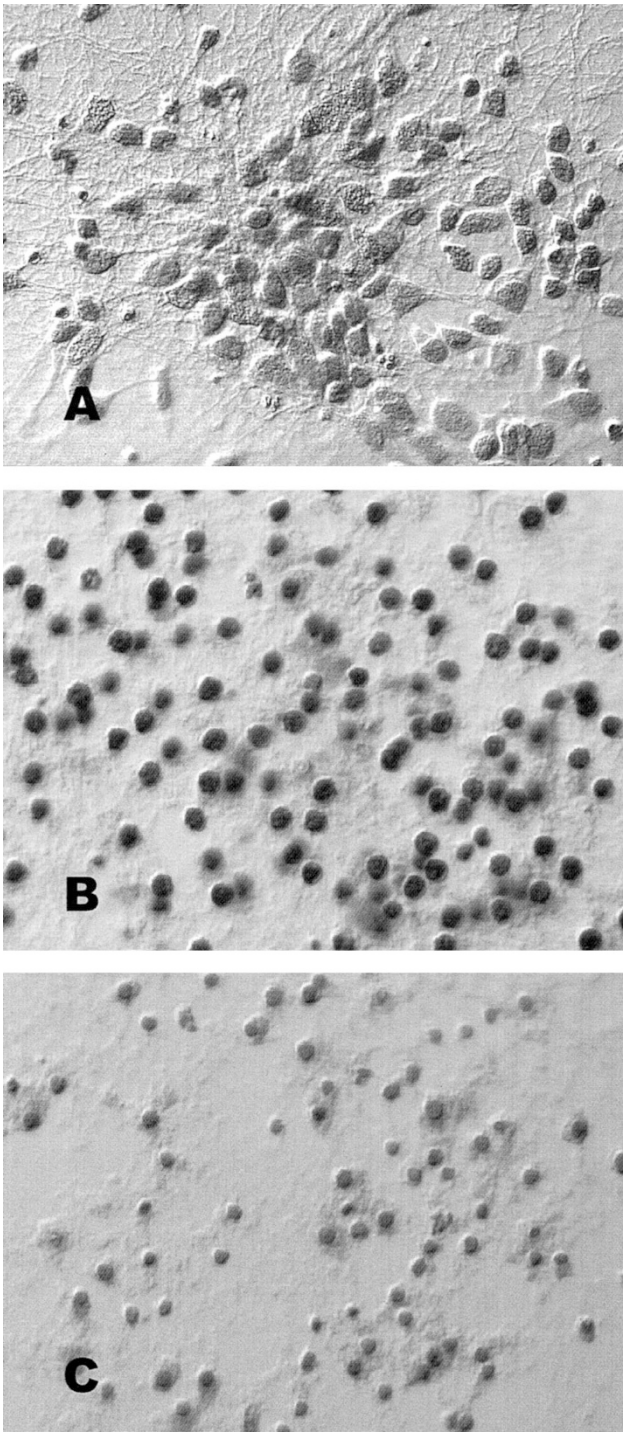

Figure 1. ISEL staining of fragmented DNA. Control cells at 24 h (*A*) and after a 60-min exposure to 100 μM (*B*) or 1 mM (*C*) of H_2O_2 and additional 24-h incubation. Light microscopy (×40, interference contrast).

At 24 h, the yields of extracted DNA from plates exposed to 100 μM and 1 mM H_2O_2 for 60 min were significantly lower than those from unexposed control cells (18.7 ± 3.0 μg and 13.4 ± 3.4 μg *versus* 39.0 ± 14.3 μg per plate, respectively, $p = 0.03$ *versus* control), indicating a corresponding loss of cells. The yield of extracted DNA of cells exposed to 10–50 μM H_2O_2 did not differ statistically from that of the unexposed control cells. These cells showed a DNA electrophoresis pattern (Fig. 2, *lane 2*) no different from that of the control cells at 24 h (Fig. 2, *lane 1*). After exposure to the intermediate concentration of H_2O_2 (100 μM), the extracted DNA was visualized as a ladder pattern suggestive of apoptosis (Fig. 2, *lane 3*). Exposure to higher (1–5 mM) concentrations of H_2O_2 resulted in smearing of the DNA (Fig. 2, *lane 4*). However, occasionally a ladder was detected (Fig. 2, *lane 5*), possibly representing fragmented DNA of residual apoptotic cells, whereas totally degraded DNA of necrotic cells would not be seen in electrophoresis or in DNA assay.

Acute AN depletion. Exposure to H_2O_2 for 60 min caused an acute dose-dependent reduction in radioactivity in total cellular ANs (Fig. 3). This depletion was accompanied by a corresponding increase of catabolic products in the culture medium,

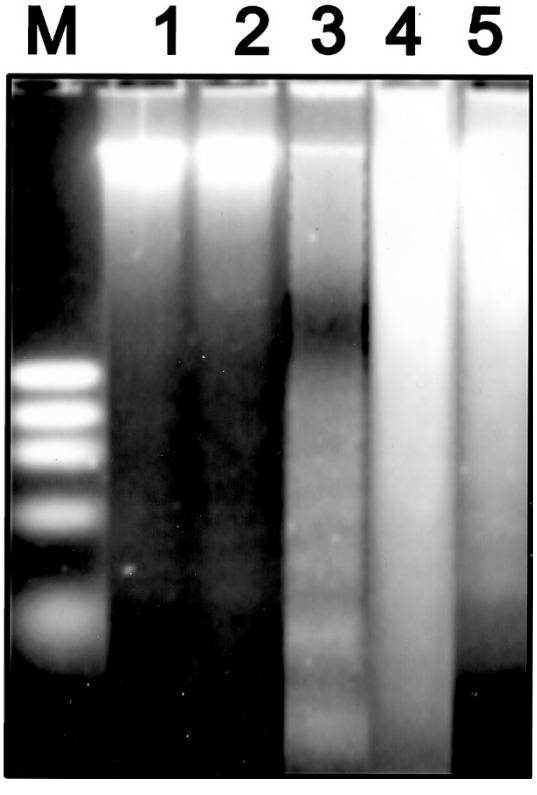

Figure 2. Fragmentation of DNA in neurons exposed for 60 min to the indicated concentrations of H_2O_2. DNA was extracted after an additional 24 h culture and separated by agarose gel electrophoresis. See text for details. *Lane 1*, control cells; *lane 2*, 10 μM; *lane 3*, 100 μM; *lanes 4* and *5*, 1 mM.

an average of 85 ± 2% of the radioactivity being in hypoxanthine. After low concentrations (10–50 μM), the cellular ANs decreased slightly but significantly to 92.0 ± 17.6% of control ($n = 16$, $p < 0.05$). An exposure to 100 μM H_2O_2 depleted cellular ANs to 48.6 ± 11.6% ($p < 0.001$ *versus* control, $n = 14$). The highest concentration of H_2O_2 tested (5 mM) caused a maximum AN depletion to 15.6 ± 6.2% of control values ($p < 0.001$ *versus* control and *versus* 1 mM exposure, $n = 10$).

Reduction in radioactivity of cellular ATP after a 60-min exposure to H_2O_2 was more pronounced than that of total ANs at intermediate to high concentrations, reflecting a corresponding decrease in cellular EC (Fig. 3). Low concentrations of H_2O_2 (10–50 μM) induced no change in ATP content, but 100 μM caused an acute depletion to 39.5 ± 11.8% ($p < 0.001$, $n = 12$), and 1 mM to 14.8 ± 3.1% of control values ($p < 0.001$ *versus* control and *versus* 100 μM exposure, $n = 6$). At higher H_2O_2 concentrations (5 mM), radioactivity in cellular ATP did not differ from blank values (3.1 ± 0.4% of control, $n = 10$).

Cellular EC. In prelabeled untreated control cell cultures, the EC, calculated from radioactivities in the separated ANs, averaged 0.83 ± 0.08. After exposure to low concentrations (10–50 μM) of H_2O_2 for 60 min the EC was 1.0 ± 0.07, which was not significantly different from control. Higher concentrations of H_2O_2 caused a dose-dependent decrease in cellular EC, reaching 38.5 ± 13.2% of control values at 5 mM H_2O_2 (Fig. 3).

Recovery and redepletion of AN levels. To assess the capacity of neurons to resynthesize and maintain their ANs after exposure to H_2O_2, the incorporation of [^{14}C]adenine into ATP was measured as a function of time and H_2O_2 concentration. These data are shown in Figure 4, which also includes cellular ATP levels immediately after exposure. ATP synthesis after exposure to 10–50 μM of H_2O_2 paralleled that of the untreated control cells, the radioactivity in cellular ATP being 92.6 ± 25.2% and 112.8 ± 14.9% of control cells at 12 and 24 h, respectively (NS *versus* control cells, $n = 12$ for 12 h, and $n = 8$ for 24 h, respectively.)

After exposure to 100 μM H_2O_2, which caused significant acute ATP depletion, the radioactivity in ATP recovered to 91.2 ± 28.0% at 12 h (NS *versus* control, $n = 12$), but showed

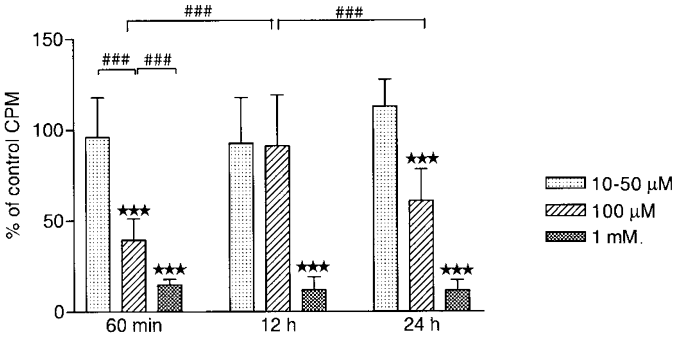

Figure 4. Cellular ATP in adenine-prelabeled cells at the end of a 60-min exposure to indicated concentrations of H_2O_2 (60 min), and in cells labeled with [^{14}C]adenine directly after exposure followed by an additional incubation for 12 h (12 h, $n = 12$) or 24 h (24 h, $n = 8$), expressed as % ± SD of control cpm. ***$p < 0.001$ *vs* control; ###$p < 0.001$ and ##$p < 0.01$ *vs* differing times or concentrations.

a secondary decline to 60.8 ± 17.5% ($p < 0.001$, $n = 12$) of untreated control values at 24 h (Fig. 4). After exposure to 1 mM H_2O_2, the radioactivity in ATP showed no recovery, remaining at 12.0 ± 7.2% and 11.6 ± 5.8% of control values, at 12 and 24 h, respectively ($p < 0.001$ *versus* control and cells exposed to 100 μM, $n = 10$ for 12 h and $n = 8$ for 24 h). Extending the incubation time to 48 h resulted in marked reduction in the uptake of [^{14}C]adenine also in untreated control cells, but the absolute counts per minute of cells treated with 100 μM of H_2O_2 continued to decline, and levels relative to control cells remained at the level of 24 h (data not shown). The incorporation of [^{14}C]adenine into ATP was in the same proportion as into the other high-energy nucleotides, so that the cellular EC levels did not differ significantly from control levels (0.92 ± 0.02) during the 12- to 24-h follow-up period at any exposure level (data not shown). Also, the radioactivity in nucleotides recovered from the medium of the exposed cells, reflecting acute cell membrane injury, remained at control values at 24 h (data not shown).

The ATP concentrations measured by HPLC corresponded well with the incorporation of [^{14}C]adenine into cellular high-energy nucleotides (Fig. 5). The cellular ATP content, normalized to protein, showed an acute reduction after treatment with 100 μM of H_2O_2 for 60 min, identical to that determined with the radioactive nucleotide depletion assay (36.5 ± 16.5% of

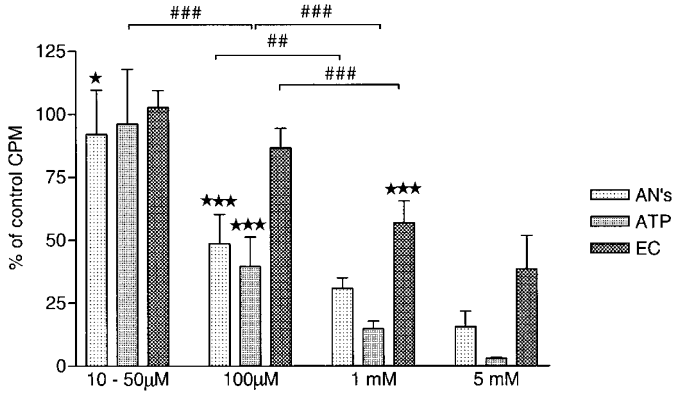

Figure 3. Cellular ANs, ATP, and EC at the end of a 60-min exposure to indicated concentrations of H_2O_2 in prelabeled cells as % ± SD of control cpm. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs* control; ##$p < 0.01$ and ###$p < 0.001$ *vs* differing concentrations. See text for details.

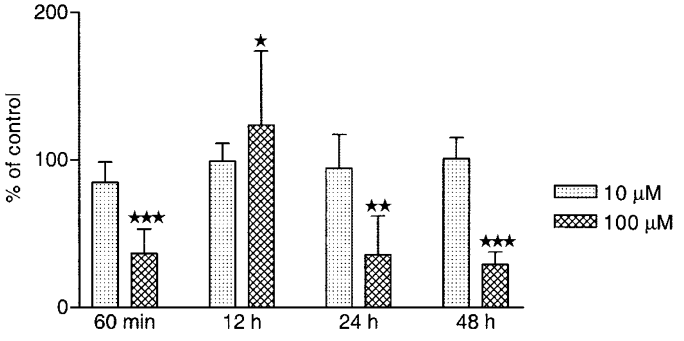

Figure 5. ATP concentration measured by HPLC and normalized to protein content as % ± SD of untreated control values at the end of a 60-min exposure to 10 or 100 μM H_2O_2 or after an additional incubation for 12 h ($n = 6$), 24 h ($n = 14$), or 48 h ($n = 4$). ***$p < 0.001$ *vs* untreated control cells.

control, $n = 6$). At low exposure level (10 μM), the ATP concentration remained at the control level up to 48 h ($n = 4$). After the intermediate insult (100 μM of H_2O_2), the acute ATP depletion recovered to 123.7 $\pm$ 50.2% of control (27.0 $\pm$ 10.4 μmol of ATP/mg of protein) by 12 h. However, at 24 h, the ATP concentration of these cells showed a secondary decline to 35.7 $\pm$ 26.1% of control, (7.3 $\pm$ 4.6 μmol of ATP/mg of protein), which continued to 29.3 $\pm$ 8.3% of control at 48 h (0.61 $\pm$ 0.2 μmol of ATP/mg of protein; Fig. 5). The ATP concentration of cells exposed to 1 mM H_2O_2 was less than the detection limit for the HPLC method at all times investigated.

DISCUSSION

We showed that H_2O_2 induces a dose-dependent reduction in cellular EC and ATP, which, after a moderate dose, temporarily recovers, but after 24 h shows a secondary reduction. This secondary depletion of cellular ATP is associated with apoptotic cell death. A mild insult causes no changes in energy metabolism or cell survival, whereas a large dose of H_2O_2 results in severe ATP depletion and necrotic cell death. Our model thus mimics the findings of *in vivo* studies in newborn animals, in which severe asphyxia followed by resuscitation produces a biphasic impairment in cerebral oxidative phosphorylation, followed by brain damage (1, 2).

Generation of reactive oxygen metabolites has been clearly documented in the reperfused brain of experimental animals (21–26). The role of oxidants in the pathogenesis of hypoxic-ischemic encephalopathy is supported by the reduction of organ damage in stroke models by antioxidant treatment (27) or by overexpression of mitochondrial superoxide dismutase (28, 29). Concentrations of H_2O_2 at the range of 100 μM have been detected *in vivo* by microdialysis during the reperfusion period after 30 min of forebrain ischemia, which results in a substantial neuronal loss at 24 h (30). *In vitro*, H_2O_2 exposure is an effective inducer of neuronal apoptosis (11), associated with acute and delayed ATP depletion (12). In neuroectodermal cells, the severity of the acute ATP depletion caused by H_2O_2 correlates with later survival and apoptosis (31).

In the present model, concentrations of H_2O_2 in the range of 10–50 μM had no or mild transient effects on neuronal ANs and caused no observable delayed damage to the cells. The primary antioxidant defense against H_2O_2 in brain is glutathione peroxidase (32), which may have adequate activity in these cultured neurons to cope with the oxidant at low concentrations. On the other hand, levels of H_2O_2 at or exceeding 1 mM caused an irreversible energy failure and acute cell death, which had the microscopic characteristics of necrosis, although a small subpopulation of cells may have survived the initial insult and died by a delayed mechanism. The intermediate concentration of 100 μM H_2O_2 caused a moderate acute depletion of ATP (to approximately 40% of control), which recovered to control levels at 12 h. Although we have no direct evidence to link this secondary increase in ATP levels to the onset of apoptosis, the ATP content in these cells may have been adequate for the death program to switch to apoptosis, which is an energy-dependent process (13, 14). The temporary recovery of ATP could also in part be ascribed to proteolytic inactivation of the ATP-consuming enzyme PARP, which is an early commitment step of apoptosis (33). By 24 h, both the uptake of ^{14}C-adenine and the ATP concentration had again declined to 61 and 36% of control values, respectively. At the time of this delayed ATP depletion, apoptotic cell death was detected by ISEL staining and DNA electrophoresis. This is in accordance with the study of Mailly *et al.* (12) in which a brief exposure to 200 μM H_2O_2 induced a decrease in neuronal ATP contents associated with increased apoptosis at 24 h.

ATP depletion may be caused by decreased synthesis, increased utilization or catabolism, or efflux out of the cells. Oxidant exposure results in rapid activation of PARP, which is followed by depletion of cellular NAD and ATP, catabolism of ANs, and decrease in EC (34). This is a likely sequence of events in our experiments. Acute oxidant exposure may also increase cellular permeability and leakage of nucleotides (35), but this only occurred at the highest H_2O_2 concentration used. During the secondary energy failure there was no significant increase in nucleotides recovered in the medium, indicative of cell membrane damage. Thus it seems possible that the secondary ATP depletion was caused by decreased synthesis. Decreased ATP synthesis, in the face of functioning adenylate kinase in the cytoplasm, would be compatible with the observation that the EC remained normal in the secondary stage of energy failure. The potential causes for decreased ATP synthesis are lack of the cosubstrate, 5-phosphoribosyl-1-pyrophosphate, for adenine salvage, or mitochondrial dysfunction, which has been shown to be associated with neuronal apoptosis induced by oxidants (29).

In addition to oxidant damage, the delayed phase of selective neuronal death after hypoxia-ischemia has been ascribed to induction of nitric oxide synthase, or activation of microglia, both of which have been shown to occur after a hypoxic insult in the CNS (9, 36). Transient exposure to H_2O_2 has also been shown to lead to accumulation of extracellular glutamate and delayed *N*-methyl-D-aspartate receptor activation (12). In our model, the roles of glutamate or activation of different forms of nitric oxide synthase have not yet been investigated.

Documentation of metabolic alterations by nuclear magnetic resonance spectroscopy gives a composite picture of events in the whole brain, but does not allow conclusions regarding different areas of the brain, or changes in neurons in comparison with other cell types. The present model aims at focusing the studies on the metabolic responses of neurons, although the possible presence of a small percentage of other cell types precludes definitive conclusions. However, the method of prelabeling the cells with a high-energy nucleotide precursor is a sensitive tool for exploring the acute and delayed effects of different exposures on cellular energy metabolism *in vitro*. The results have, of course, limited analogy to the complex interactions occurring in the CNS during ischemia-reperfusion. This model may prove useful for further investigating the roles played by PARP activation or inactivation, inducible nitric oxide synthase induction, or modifications caused by neuronal-glial interactions. It also allows exploration of the possibilities of preventing the secondary deterioration of neuronal energy metabolism after an oxidative insult.

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