Increased Hexokinase Activity, of Either Ectopic or Endogenous Origin, Protects Renal Epithelial Cells against Acute Oxidant-induced Cell Death*

Glucose (Glc) metabolism protects cells against oxidant injury. By virtue of their central position in both Glc uptake and utilization, hexokinases (HKs) are ideally suited to contribute to these effects. Compatible with this hypothesis, endogenous HK activity correlates inversely with injury susceptibility in individual renal cell types. We recently reported that ectopic HK expression mimics the anti-apoptotic effects of growth factors in cultured fibroblasts, but anti-apoptotic roles for HKs have not been examined in other cell types or in a cellular injury model. We therefore evaluated HK overexpression for the ability to mitigate acute oxidant-induced cell death in an established epithelial cell culture injury model. In parallel, we examined salutary heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) treatment for the ability to 1) increase endogenous HK activity and 2) mimic the protective effects of ectopic HK expression. Both HK overexpression and HB-EGF increased Glc-phosphorylating capacity and metabolism, and these changes were associated with markedly reduced susceptibility to acute oxidant-induced apoptosis. The uniform Glc dependence of these effects suggests an important adaptive role for Glc metabolism, and for HK activity in particular, in the promotion of epithelial cell survival. These findings also support the contention that HKs contribute to the protective effects of growth factors.

Glucose (Glc) metabolism plays a critical role in the protection of a variety of cell types against oxidant-induced cell death.

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1 The abbreviations used are: Glc, glucose; DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; HBSS, Hank’s balanced salt solution; HK, hexokinase; HA PT, Homo sapiens proximal tubule cells; LDH, lactate dehydrogenase; m.o.i., multiplicity of infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetra-

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changes in endogenous HK activity could contribute to the well-described salutary effects of growth factors on this cell type (22). Heparin-binding EGF-like growth factor (HB-EGF) was specifically selected for study on the basis of its increased renal expression during recovery from acute ischemic injury (23), as well as the demonstrated ability of the closely related family member EGF to ameliorate toxic and ischemic renal injury in vivo (22, 24–26). These findings have led some investigators to propose protective and/or reparative roles for HB-EGF in the oxidant injury associated with renal ischemia-reperfusion (22, 23), so we directly tested this growth factor for the ability to both increase endogenous proximal tubule cell HK activity and mimic the protective effects of HK overexpression. In parallel, individual protective effects associated with either ectopic or endogenous HK activity were specifically examined for dependence upon the availability of Glc.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All cell culture reagents, including serum and media additives, were supplied by Invitrogen (Grand Island, NY). Grade I yeast Glc 6-phosphate dehydrogenase and a lactate dehydrogenase (LDH) activity detection kit were purchased from Roche Molecular Biochemicals (Indianapolis, IN). The chemical fluorophores 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and YO-PRO-1 were obtained from Molecular Probes (Eugene, OR). All other reagents, including NADPH, NADP, and recombinant human HB-EGF, were from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

**Cell Culture**—Mycoplasma-free HsPCTC (Homo sapiens Proximal Tubule Cells) were obtained from the American Type Culture Collection (Rockville, MD) at passage 15. These cells are clonally derived from normal adult human proximal tubule cells that have been immortalized by transduction with human papillomavirus (HPV 16) E6 and E7 genes (19). They exhibit both biochemical and morphological features of normal proximal tubule cells in culture (19, 27) and represent an established cell culture model of oxidant-induced proximal tubule injury (19–21). Cells were routinely grown in monolayer culture on polystyrene dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) or uncoated glass chamber slides (LabTek II, Nalge Nunc, Naperville, IL). Normal growth medium consisted of standard Dulbecco’s modified Eagle Ham’s F-12 (1:1) medium supplemented with 5% fetal bovine serum as described previously (27) but with the following modifications: The medium was buffered with 20 mM HEPES, and the Glc content was reduced to 7.5 mM. Cells were maintained at 37 °C in a humidified 5% CO2 incubator, and all experiments were performed between passages 15 and 30 to minimize the effects of phenotypic variation in continuous culture. Cells were passaged routinely serum-deprived for 16–24 h to induce quiescence prior to testing.

**Adenoviral Transgene Delivery and Expression**—Recombinant replication-deficient adenoviruses expressing HKI (rAd-HKI), the GLUT1 facilitative Glc transporter (rAd-GLUT1), and a β-galactosidase reporter gene (rAd-LacZ) (28) were obtained from Dr. Christopher B. Newgard (University of Texas Southwestern Medical Center, Dallas, TX) and were used to transfect quiescent HsPCTC monolayers as described previously (29). Ectopic HK expression was monitored by coupled enzymatic HK assays (see below), and β-galactosidase expression in paired LacZ-transfected control cells was monitored by a standard chromogenic assay as described previously (31, 32) with minor modifications. The final assay mixture consisted of 1 unit/ml Glc 6-phosphate dehydrogenase, 0.5 mg/ml β-NADP, 6.7 mM ATP, 7 mM MgCl2, 4 mM Glc, 2.5 mM KH2PO4, 1 mM NaH2PO4, 11.1 mM monothioglycerol, 0.01% (v/v) Triton X-100, 25 μM EDTA, and 45 mM Tris-HCl, pH 8.5. Total cellular protein content was assessed by the method of Bradford (33) using bovine γ-globulin (Bio-Rad) as a reference standard, and all data were expressed as specific HK activity in units per g of total protein, where 1 unit is defined as that amount of enzyme activity resulting in the coupled formation of 1 μmol of NADPH per min at 25 °C.

**Metabolic Assays**—Glc utilization and lactate production were evaluated as described previously (31, 32) and were assayed as the net rates of Glc disappearance and lactate accumulation in the culture medium, respectively. For these experiments, cells were cultured in growth medium containing 7.5 mM Glc and lacking both serum and phenol red. Where appropriate, medium aliquots were assayed colorimetrically for both Glc and lactate content. Net cellular reductive capacity was also monitored using a commercially available tetrazolium reduction assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega, WI). This assay, which is based on the ability of viable cells to reduce MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (34)) in the presence of the electron acceptor phenazine ethosulfate, is widely employed to monitor net cellular reductive capacity as an indicator of both proliferation and viability. Originally attributed to mitochondrial metabolism (35), it has subsequently been demonstrated that tetrazolium bioreduction to yield formazan chromophores is primarily extramitochondrial and largely involves the pyridine dinucleotides NADH and NADPH (36). It has also been shown that formazan generation is critically dependent upon both the uptake and continued metabolism of Glc (37, 38), presumably via the coupled reduction of pyridine dinucleotides. Thus, chromophore formation will necessarily reflect both ongoing metabolic and oxidative consumption of reduced nicotinamide co-factors. MTS reduction assays were performed according to the manufacturer’s recommendations and were exploited to monitor net cellular reductive capacity in real-time as described previously (38).

**Acute Oxidative Stress Model**—The oxidative stress model used for these studies was essentially as described by Zager and colleagues (19–21) with minor modifications. HsPCTC cells were routinely grown to ~90% confluence in normal growth medium before serum deprivation overnight to induce quiescence. In preliminary testing, serum deprivation alone, for periods up to 24 h, was not sufficient to induce apoptosis in these cells.3 Quiescent cell monolayers were then exposed to H2O2 at concentrations up to 10 mM for as long as 3 h at 37 °C in Hanks’ balanced salt solution (HBSS) containing physiologic levels of Glc (5.6 mM).

**Cell Viability-Death Assays**—In initial experiments, cell death was monitored microscopically by the uptake of both YO-PRO-1 (39) and PI, fluorescent markers of apoptosis and necrosis, respectively. Quiescent HsPCTC monolayers were exposed to H2O2 (0–10 mM) for up to 3 h at 37 °C in HBSS containing 1 μM YO-PRO-1 and/or 1.5 μM PI. The cellular uptake of both fluorophores was monitored, alone and in combination, using a Carl Zeiss LSM 410 laser scanning confocal microscope equipped with an argon/krypton laser as described (40), and unstressed control cells were routinely analyzed in parallel to exclude direct toxic effects of the fluorophores. The exclusion of both YO-PRO-1 and PI from viable, non-apoptotic cells makes them useful to screen for cell death in real-time, but because not all cells are visualized by this method, additional measures are required for quantitative estimates of cell death. For this reason, quantitative assessment of apoptosis was performed under selected conditions by conventional DAPI staining and cell counting (29, 41). In these experiments, cells were fixed, where appropriate, by the direct addition of formaldehyde to the medium to a final concentration of 10% (v/v). Detached cells were allowed to settle and adhere to the slide before DAPI staining and analysis using a Zeiss Axiosplan 2 fluorescence microscope system equipped with a matched ultraviolet light source. Apoptotic cells were manually counted and expressed as a percentage of the total cell number. At least four independent fields of ~100 cells were scored for each experimental condition.

**Cytolysis Assays**—Plasma membrane integrity was serially assessed by monitoring cytotoxic lactate dehydrogenase (LDH) release into the culture medium using a commercially available assay kit (Roche Molecular Biochemicals) per the manufacturer’s recommendations. In brief, LDH activity was colorimetrically assayed in cell-free aliquots of culture supernatants via the coupled enzymatic reduction of a tetrazolium salt to generate a formazan chromophore. The total releasable pool of cellular LDH activity was always assayed in identical paired cell monolayers following lysis with 1% (v/v) Triton X-100, and the proportion of total activity in the supernatant was taken as an index of the degree of cell lysis.

**Statistical Analysis**—All data are presented as the means ± S.E. for at least three independent experiments. Statistical comparisons were performed by two-tailed paired t-testing using a significance level of

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2. Originally designated HK-2 cells, this nomenclature is not used in the present work to avoid confusion with the HKII isozyme.

3. J. M. Bryson and R. B. Robey, unpublished observations.
RESULTS

H₂O₂ Decreases Both HsPTC Viability and Net Cellular Reductive Capacity in a Time- and Concentration-dependent Manner—To characterize the general response of HsPTC to acute oxidative stress, quiescent cell monolayers were exposed to varying concentrations of H₂O₂ (0–10 mM) in the presence of fluorescent indicators of apoptosis (1 μM YO-PRO-1) and/or necrosis (1.5 μM PI). H₂O₂ increased the cellular uptake of both fluorophores in a concentration- and time-dependent manner, with virtually all cells exhibiting YO-PRO-1 uptake and nuclear condensation characteristic of apoptosis within 2 h of exposure to 10 mM H₂O₂ (Fig. 1A). PI uptake exhibited similar H₂O₂ concentration dependence but typically lagged behind YO-PRO-1 uptake by 0.5–1.0 h. In addition to this temporal delay, PI uptake by cells incubated with both fluorophores was observed only in YO-PRO-1-staining apoptotic cells, suggesting the development of secondary necrotic changes in these cells. Acute oxidative stress was also associated with marked depression of in situ tetrazolium bioreduction (Fig. 1B). This effect was similarly concentration-dependent and was most marked at H₂O₂ concentrations associated with increased early apoptosis (Fig. 1A). Changes in net cellular reductive capacity, however, uniformly preceded the uptake of both YO-PRO-1 and PI, which is temporally compatible with roles for both metabolism and cellular redox status in this model of acute oxidant-induced cell death.

Adenoviral HKI Transgene Delivery Increases Total HK Activity in a Titer-dependent Manner—To directly assess the functional consequences of primary increases in cellular Glc phosphorylating capacity, HsPTC were transfected with recombinant adenoviruses expressing either a β-galactosidase reporter gene (rAd-LacZ) or an HKI transgene. As depicted in Fig. 2A, control adenoviral transfections using the β-galactosidase-expressing vector (rAd-LacZ) resulted in uniform, titer-dependent expression of β-galactosidase reporter gene (rAd-HKI; Fig. 2B) similarly increased total HK activity in a titer-dependent manner. Basal HK activity (17 ± 1 units/g of protein) in untransfected HsPTC was comparable in magnitude to that reported previously for both this cell line (27) and for primary cultures of human proximal tubule cells (42). In paired cell monolayers, infection with rAd-HKI at a relative multiplicity of infection (m.o.i.) of ~1 increased total HK activity at 24 h by over 40% (25 ± 2 units/g of protein, p < 0.001), whereas a 10-fold higher viral titer increased total HK activity nearly 4-fold (64 ± 7 units/g of protein, p < 0.001).

Cells Expressing Ectopic HKI Exhibit Decreased Susceptibility to Acute Oxidant-induced Cell Death—β-Galactosidase-expressing (+rAd-LacZ), recombinant HKI-expressing (+rAd-HKI), and paired untransfected HsPTC cell monolayers were acutely exposed to H₂O₂ over a concentration range (1–10 mM) previously required to induce rapid oxidant-induced death in both these cells (19–21) and isolated proximal tubule segments (21, 43). As shown in Fig. 3, cells overexpressing HKI (+rAd-HKI, m.o.i. ~1; lower panels) exhibited improved cell morphology and attachment, as well as decreased uptake of both YO-PRO-1 and PI, relative to paired untransfected controls (wt; upper panels) or cells expressing a β-galactosidase reporter gene (+rAd-LacZ, m.o.i. ~1; middle panels) when exposed to 10 mM H₂O₂ for 3 h. Qualitatively similar effects were observed at lower H₂O₂ concentrations, but they were temporally delayed and markedly extended the time frame required for analysis. The salutary benefits of ectopic HK expression were associated with modest increases in total HK activity (>40%) that were comparable in magnitude to those previously associated with reduced apoptosis in cultured fibroblasts following prolonged growth factor withdrawal (29).
Cytotoxicity of Hexokinase Activity

Additional protective benefits were not observed at the higher levels of HK activity associated with higher rAd-HKI titers, so all subsequent experiments were conducted at comparable functional titers (i.e., m.o.i. ~1), except where noted. The representative experiment depicted in Fig. 3 was repeated three times with identical results.

HK Overexpression Decreases Cytolysis in Both STRESSED and UNSTRESSED HSPTC—We also monitored the extracellular release of cytotoxic LDH as a marker of plasma membrane integrity. Total cellular LDH content in these experiments remained stable and was not different in paired stressed and unstressed cells. Basal LDH release by unstressed cells averaged 10 ± 1% of the total cellular LDH content over 3 h. As depicted in Fig. 4, exposure to 1 mM H2O2 nearly doubled the rate of LDH release during this time period, whereas exposure to 10 mM H2O2 more than trebled this response (*, p < 0.05). These findings were both temporally and quantitatively compatible with the observed changes in cellular PI uptake by oxidant-stressed HSPTC. Ectopic HK expression by rAd-HKI prevented the increase in LDH release by cells exposed to 1 mM H2O2 in a titer-dependent manner (Fig. 5). Interestingly, HKI overexpression also decreased LDH release by unstressed cells, suggesting small, albeit significant (*, p < 0.05), salutary effects on basal apoptosis as well.

Ectopic HKI Expression Exerts Anti-APoptotic Effects That Correlate with Increased Cellular Glc Phosphorylating Capacity—Apoptotic cell death was quantitatively assessed in DAPI-stained HSPTC by microscopic examination and manual counting of condensed and fragmented nuclei (29, 41). Both stressed (10 mM H2O2) and unstressed control monolayers were routinely scored for apoptosis in parallel with β-galactosidase-, HKI-, or GLUT1-overexpressing cells. As shown in Fig. 6A, exposure of unstressed HSPTC to 10 mM Glc in the presence of 7.5 mM Glc more than doubled the number of apoptotic cells at 1 h. Ectopic HK expression (+ rAd-HKI) largely prevented this increase (*, p < 0.05), in agreement with the corresponding decrease in YO-PRO-1 uptake described above. In contrast, both β-galactosidase reporter gene expression (+ rAd-LacZ) and facilitative Glc transporter overexpression (+ rAd-GLUT1) failed to mimic the effects of ectopic HK expression on the fractional rate of oxidant-induced HSPTC apoptosis. It is pertinent to note, however, that, in individual experiments, GLUT1-overexpressing cells consistently exhibited oxidant-induced apoptotic rates that were intermediate between those observed for HKI-overexpressing cells and β-galactosidase-expressing control cells. Pairwise analysis of the data at 1 h confirmed that apoptosis increased by 209 ± 50% (p < 0.02 versus unstressed controls) and 206 ± 49% (p < 0.02 versus unstressed controls) in β-galactosidase-expressing and untransfected control cells, respectively. These responses were indistinguishable from one another (p = 0.93), whereas apoptosis in oxidant-stressed HKI-overexpressing cells increased by only 53 ± 12% (p < 0.05 versus oxidant-stressed β-galactosidase-expressing and untransfected controls). The corresponding response in GLUT1-overexpressing cells (125 ± 30%) was intermediate between these extremes but never achieved statistical significance in these studies (p = 0.17 and p = 0.10 versus β-galactosidase-expressing and untransfected control cells, respectively). As shown in Fig. 6B, total HK activity (17 ± 2 units/g of protein in untransfected HSPTC) was increased by HKI overexpression in these studies (24 ± 2 units/g of protein, p = 0.0003) but was not affected by either β-galactosidase reporter gene expression (17 ± 1 units/g of protein, p = 0.72) or ectopic GLUT1 expression (18 ± 2 units/g of protein, p = 0.26).

The Salutary Effects of Ectopic HK Expression Require the Availability of Glc—To examine whether the protective effects associated with increased HK activity depend upon substrate availability, we specifically examined the ability of rAd-HKI to mitigate acute oxidant-induced HSPTC apoptosis in both the presence and absence of Glc. As shown in Fig. 7, Glc removal from the medium during exposure to 10 mM H2O2 abrogated the cytotoxic effect of ectopic HKI expression. In addition, the rate of oxidant-induced apoptosis was actually enhanced in both untransfected and HKI-overexpressing HSPTC in the absence of Glc, suggesting intrinsic Glc-dependent protective mechanisms that presumably include endogenous HK activity.

HB-EGF Increases Endogenous HK Activity and Mimics the Glc-dependent Effects of Ectopic HK Expression—To address the hypothesis that endogenous HK activity could contribute to the salutary effects of growth factors, we first examined the ability of HB-EGF to increase endogenous HK activity and Glc metabolism in HSPTC. In preliminary experiments, HB-EGF increased total HK activity in a concentration- and time-dependent manner, with maximal HK induction observed within 12–24 h of exposure to ≥1 nM HB-EGF (apparent ED50 ~1 nM). As shown in Fig. 8A, 10 nM HB-EGF increased HK activity by over 25% (21.0 ± 1.1 versus 16.6 ± 1.1 units/g of protein; p < 0.03) at 24 h, and Fig. 8B demonstrates that these changes were associated with ~50% net increases in both Glc utilization (3.9 ± 0.2 versus 2.8 ± 0.2 mmol/g of protein/h; *, p < 0.03) and lactate accumulation (6.3 ± 0.5 versus 4.1 ± 0.3 mmol/g of protein/6 h; *, p < 0.002). We then examined whether these changes were associated with altered susceptibility to acute oxidant-induced cell death. In the absence of HB-EGF pretreatment, 10 mM H2O2 more than doubled the number of apoptotic

FIG. 2. Adenoviral HKI transgene delivery increases total HK activity in a concentration-dependent manner. Confluent cell monolayers were routinely transfected with matched recombinant pJM17-based adenoviruses (28) expressing a β-galactosidase reporter gene (rAd-LacZ) or a rat HKI transgene (rAd-HKI). As shown in A, transfections with rAd-LacZ resulted in uniform, titer-dependent reporter gene expression, with virtually all cells expressing cytochemically detectable β-galactosidase at viral multiplicities-of-infection (m.o.i.) ≥1. A representative set of control transfections is shown. As depicted in B, transfection with rAd-HKI resulted in similar titer-dependent HKI gene expression. At an m.o.i. ~1, rAd-HKI increased total HK activity by over 40% at 24 h (*, p < 0.001). Exposure to a 10-fold greater titer of rAd-HKI (m.o.i. ~10) resulted in a corresponding 4-fold increase in total HK activity (*, p < 0.001). Higher viral titers were associated with much smaller increments in HK activity and were variably accompanied by evidence of cellular toxicity, ostensibly due to higher levels of native adenoviral gene product expression (29).
cells within 40 min (Fig. 8C; *, $p < 0.005$ versus unstressed control cells), and these results are consistent with the corresponding responses depicted in Figs. 6A and 7. In contrast, pretreatment of paired cell monolayers with 10 nM HB-EGF for 24 h completely prevented this increase in acute oxidant-induced apoptosis (Fig. 8D; †, $p = 0.02$ and ‡, $p < 0.004$ versus time-paired HB-EGF-naïve control cells). As shown in Fig. 9, the protective effect of HB-EGF pretreatment was also fully abolished by the removal of Glc from the media, suggesting similar Glc dependence and a requirement for metabolism. All other factors being equal, the rates of oxidant-induced apoptosis were uniformly higher in the absence of Glc, which is compatible with the results presented in Fig. 7 and suggests intrinsic Glc-dependent protective mechanisms in these cells.

**DISCUSSION**

In this study, we have clearly shown that primary increases in HK activity, vis-à-vis ectopic HK expression, are associated with decreased renal epithelial cell susceptibility to oxidant-induced cell death. In parallel, we have demonstrated that HB-EGF and associated increases in endogenous HK activity have similar cytoprotective benefits. The uniform Glc dependence of these effects suggests a metabolic requirement for substrate availability and is compatible with the contention that they require Glc phosphorylation by HKs. Coupled with our previous findings (29), these results are also compatible with the hypothesis that HKs play an important role in the anti-apoptotic effects of growth factors.
Cytotoxic and cytolysis by H2O2, Hs

Fig. 4. H2O2 increases HsPTC lysis in a concentration-dependent manner. Acute oxidant-induced cell lysis was monitored as the release of cytosolic LDH into the culture medium. Basal LDH release by unstressed cells was consistently ~10% of the total cellular LDH content over this period and was comparable in magnitude to basal levels of cell lysis reported for isolated rat proximal tubules (43). An increase of ~3-fold in LDH release was observed within 3 h in the presence of 10 mM H2O2, and intermediate responses were observed in the presence of 1 or 5 mM H2O2 (*, p < 0.02 versus basal).

Fig. 5. Ectopic HK expression increases cell viability in both stressed and unstressed HsPTC. As demonstrated in Fig. 4, total LDH release by cells exposed to 1 mM H2O2 for 3 h (•) was nearly 2-fold higher than that associated with paired, unstressed control cells (○, p < 0.02). However, prior transfection with rAd-HKI prevented oxidant-induced cytolysis in a titer-dependent manner and increased cellular viability in unstressed control cells as well (*, p < 0.05 versus paired oxidant-stressed controls). All data represent the means ± S.E. for at least three independent experiments, and error bars are depicted were they are larger than the symbols used to depict the means.

Because cell death cannot be fully defined by, or ascribed to, a single biochemical parameter, we monitored multiple indices of cell viability and integrity following severe acute oxidative stress. Identical results obtained using multiple independent measures validated the ability of increased HK activity to prevent acute oxidant-induced cell death. Ectopic HK expression was associated with improved cell morphology, decreased cell detachment, decreased apoptosis, and reduced cytolysis in H2O2-stressed cells. Rapid decreases in the net reductive capacity of HsPTC preceded the appearance of markers of apoptosis and cell death. As the earliest demonstrable response to acute oxidative stress, these changes suggest an antecedent imbalance between reductive consumption and production. Given the dependence of tetrazolium bioreduction upon Glc metabolism (37, 38), they are fully compatible with a role for Glc metabolism in these responses. Characteristic apoptotic changes also uniformly preceded the loss of plasma membrane integrity in oxidant-stressed cells, suggesting that cytolysis may represent a secondary effect in these cells. These findings are consonant with our previous demonstration that ectopic HK expression promotes cell survival by preventing early apoptotic events following growth factor withdrawal (29). Enhanced oxidant-induced apoptotic responses in both untransfected and HKI-overexpressing HsPTC in the absence of Glc are also suggestive of intrinsic Glc-dependent protective mechanisms that presumably involve endogenous HK activity. Indirect support for cytoprotective roles for HKs may also be found in the reported association between HKI expression and recovery of ventricular function in a rat myocardial infarction model (44).

Although facilitative Glc transporter overexpression failed to fully mimic the effects of ectopic HK expression, our findings are not incompatible with previous reports of salutary effects associated with Glc transporter overexpression (8, 45), and they do not exclude a role for endogenous Glc transporters in our findings. Major control over cellular Glc uptake and utilization is shared between both transport and phosphorylation (46), and because metabolic control may be variably distributed
between these functionally interactive processes under differing cellular conditions (47), it is likely that Glc transport plays an important role in our findings. However, the inability of GLUT1 overexpression to functionally substitute for ectopic HK expression clearly suggests that substrate entry alone is not sufficient to protect cells from acute oxidant-induced cell death. These findings are compatible with previously demonstrated requirements for Glc metabolism in cellular recovery from oxidative post-hypoxic injury (2, 4, 6, 8). Based upon both the present findings and our previous studies in fibroblasts (2, 4, 6, 8), Glc plays a major role in the cytoprotective effects of HB-EGF pretreatment. To directly address the substrate dependence of the protective effects of HB-EGF, we examined HB-EGF-pretreated cells (10 nM HB-EGF for 24 h) for susceptibility to acute oxidant-induced apoptosis in both HKI-overexpressing cells (6, 8) and untransfected cells (6, p > 0.59), suggesting the complete dependence of HK-associated protective effects on substrate availability. All data represent the means ± S.E. for at least three independent experiments, with the following exception: Data depicted at 1.5 h (∞) represent results from a pair of independent experiments used to define the curves beyond 1 h but not included in the statistical analysis of the data because of sample size limitations. Error bars are shown where they are larger than the symbols used to depict the means.

FIG. 7. The salutary effects of ectopic HK expression require substrate availability. As demonstrated in Fig. 6A, HKI overexpression (+rAd-HKI) prevented oxidant-induced apoptosis in the presence of 5.6 mM Glc (■, *, p < 0.03 versus paired untransfected control cells, ●). Glc removal from the medium not only abrogated the protective effect of HKI overexpression, it enhanced the rate of oxidant-induced apoptosis in both HKI-overexpressing cells (○, ♦, p < 0.04 and ★, p = 0.007 versus HKI-overexpressing cells in the presence of Glc, ■) and untransfected control cells (□, ♦, p < 0.03 versus corresponding cells in the presence of Glc, ●). In the absence of Glc, oxidant-induced apoptosis was indistinguishable between HKI-overexpressing cells (○) and untransfected cells (□, p > 0.59), suggesting the complete dependence of HK-associated protective effects on substrate availability. All data represent the means ± S.E. for at least three independent experiments, with the following exception: Data depicted at 1.5 h (∞) represent results from a pair of independent experiments used to define the curves beyond 1 h but not included in the statistical analysis of the data because of sample size limitations. Error bars are shown where they are larger than the symbols used to depict the means.

FIG. 8. HB-EGF pretreatment mimics the effect of ectopic HK expression. As shown in A, HB-EGF pretreatment with 10 nM HB-EGF increased total HK activity at 24 h (*, p < 0.03), and, as demonstrated in B, this increase was temporally associated with net increases in both Glc utilization and lactate accumulation (●, + HB-EGF; ○, − HB-EGF; ♦, p < 0.03 and ★, p < 0.002, respectively). C, in the absence of HB-EGF pretreatment, 10 nM H2O2 increased apoptosis normally (●, *, p < 0.005 versus untransfected control cells, ○). In D, it can be appreciated that HB-EGF pretreatment (●) completely prevented this acute increase in oxidant-induced apoptosis (†, p = 0.02 and ★, p < 0.004 versus corresponding time-paired HB-EGF-naïve cells; see C). In contrast, HB-EGF had no corresponding independent effects on basal apoptosis in untransfected HsPTC (■).

FIG. 9. The cytoprotective effects of HB-EGF pretreatment are also Glc-dependent. To directly address the substrate dependence of the protective effects of HB-EGF, we examined HB-EGF-pretreated cells (10 nM × 24 h) for susceptibility to acute oxidant-induced apoptosis in both the presence and absence of Glc. Consistent with the results shown in Fig. 8, HB-EGF pretreatment decreased acute oxidant-induced apoptosis in the presence (●, *, p < 0.05) but not the absence (○) of Glc. Rates of apoptosis were uniformly higher in the absence of Glc (○, ■) when compared with identical cells with access to physiologic levels of Glc (●, ■), consistent with the presence of intrinsic Glc-dependent protective mechanisms.

may energetically favor cell survival by enhancing glycolytic flux. The importance of cellular ATP content in cell viability is widely accepted (6, 50), and Glc plays a major role in the

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maintenance of intracellular ATP. The widespread use of Glc removal and glycolytic inhibitors in ATP depletions models of proximal tubule injury constitutes tacit acknowledgment of the importance of Glc metabolism to survival in this cell type. Lastly, HKs, specifically the HKI and HKII isoforms, are distinguished from all other glycolytic enzymes by their capacity to physically associate with mitochondria and directly couple the first committed step of Glc metabolism to oxidative phosphorylation (29, 51–53). Mitochondria play a pivotal role in apoptotic cell death, and we have recently shown that mitochondrial HK association promotes both mitochondrial integrity and cell survival following growth factor withdrawal, independent of downstream glycolytic flux (29). Given the inherent complexities of metabolic regulation, it is possible, and even likely, that other consequences of metabolism (e.g., pH) contribute to the protective effects of Glc. An exhaustive examination of these individual possibilities, however, is beyond the scope of the present work and remains for future studies to address.

The exogenous H2O2 concentrations used for these studies may exceed those anticipated in vivo. They are, however, comparable in magnitude to those employed previously in both the characterization of this cell line and its validation as a cell culture model of oxidant injury (19–21). They are also comparable to the minimum H2O2 concentrations reported to consistently induce rapid cell death in isolated rat proximal tubular segments in the presence of physiologic concentrations of Glc (43). Because oxidant-induced cell death will necessarily reflect the balance between incident stress and intrinsic protective mechanisms, it is reasonable to assume that the observed salutary effects of increased HK activity under conditions of severe acute oxidative stress would also have implications for lesser degrees of stress. Our observations of qualitatively similar, albeit temporally delayed, responses to lower concentrations of H2O2 are compatible with this inference. It is also important to note that the conditions selected for study in the present work greatly facilitated the examination of these effects by increasing the signal-to-noise ratio and by compressing the time frame for analysis, thereby avoiding the confounding pro-apoptotic influence of prolonged culture (>24 h) in the absence of serum.

In conclusion, we have shown that increased HK activity, of either ectopic or endogenous origin, is associated with decreased HsP70 susceptibility to oxidant-induced cell death. The effective changes in HK activity were comparable in magnitude to those previously shown to mimic the anti-apoptotic effects of growth factors in cultured fibroblasts (29). They are also similar to reported changes in endogenous HK activity both in vivo (11–14, 54, 55) and in a variety of cell culture models (31, 32, 56).6 HB-EGF expression is increased in a wide variety of renal injury models, including ischemia-reperfusion injury where both protective and reparative roles have been proposed for this growth factor (22, 23). It is therefore of considerable interest that HB-EGF mimicked the cytoprotective effects of ectopic HK expression and was associated with increased endogenous HK activity and Glc metabolism. These findings suggest metabolic adaptations relevant to the study of ischemia-reperfusion injury, as well as the intriguing possibility that increased HK activity may contribute to the salutary effects of growth factors in the kidney (22). Indirect support for this hypothesis may be found in recent descriptions of Glc-dependent interleukin-3-mediated hematopoietic cell survival (45, 57, 58) and an associated decrease in HK expression in these cells following growth factor withdrawal (58). Although the Glc dependence of other anti-apoptotic effects of growth factors has not been fully examined, the present work, coupled with our previous findings (29), could suggest a general role for both HK activity and Glc metabolism in growth factor-mediated survival in diverse cell types. As a corollary of these hypotheses, HKs may constitute potential therapeutic targets to prevent or mitigate acute oxidant renal injury. Lastly, it would be attractive to speculate that adaptive changes in HK activity in normal cells may have a maladaptive counterpart in cancer cells, where glycolytic capacity is known to correlate with both proliferative capacity and cell survival. Markedly increased HK expression and activity are central features of the classical biochemical phenotype of tumor cells detailed by Warburg (59) in the early part of the last century. These changes also constitute the principal biochemical basis for the use of positron emission tomographic imaging of fluorodeoxyglucose uptake to detect tumor metastases (60). Taken together, it is reasonable to postulate that increased HK activity may contribute to the ability of a wide range of cell types to evade programmed cell death. Thus, our findings not only suggest mechanisms whereby growth factors may exert their salutary effects on acute oxidant injury but also have specific physiologic, pathophysiologic, and possibly therapeutic implications.

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