Abstract. Reactive oxygen species (ROS) are important mediators for several biologic responses, including apoptosis. The present study evaluated the time course of changes in intracellular ROS production and apoptosis-related proteins, as well as apoptotic changes in human tubular proximal cells (HK-2 cells) exposed to hyperglycemia. Apoptosis (annexin V binding), ROS formation (fluorescence probe dichlorofluorescin diacetate and FACScan flow cytometry), and X chromosome–linked protein (XIAP; Western blot) were studied in HK-2 cells grown in a medium containing normal (NG) or high glucose (HG) concentrations (5.5 or 30 mM, respectively) for 18 to 48 h. HG promoted an increase (65% at 18 h and 73% at 24 h; P < 0.05 versus NG) in intracellular ROS generation. At 18 h, the NF-kB binding activity (evaluated by electrophoretic mobility-shift assay) was suppressed by HG. At the same time, the expression of NF-kB–induced antiapoptotic XIAP was reduced in HG-treated cells. Apoptotic changes were observed at 48 h (34 ± 7% in HG versus 10 ± 3% in NG; P < 0.001). Changes in ROS production at 24 h predicted changes in the apoptotic index at 48 h (r = 0.96, P < 0.0001). These results suggest that hyperglycemia induces apoptotic changes in human tubular cells via an increase in oxidative stress and that a downregulation of antiapoptotic protein XIAP is a component of this response.

Most studies on diabetic nephropathy have focused on functional and structural changes in the glomeruli. However, alterations in tubular function have been observed as an early phenomenon in diabetes (1). Moreover, changes within the tubulointerstitium occur in a large subset of patients with diabetic nephropathy and play a major role in determining the renal outcome (2). The mechanisms by which diabetes leads to tissue damage and contributes to the development of tubulointerstitial damage are not understood. Recent observations indicate that hyperglycemia triggers the generation of free radicals and oxidant stress (3) in both mesangial (4) and tubular cells (5,6). Reactive oxygen species (ROS) are considered to be important mediators for several biologic responses, including proliferation, extracellular matrix deposition, and apoptosis (7). Although in the past ROS have been regarded as toxic metabolites, this concept has been revised in consideration of recently proposed roles for ROS as second messenger (4). It has been suggested that cell-type specificity does occur as for signaling events by which cells respond to ROS, thus explaining the different response in different cell types (4).

Apoptosis might contribute to the structural changes that occur in the diabetic kidney (8). We have previously observed that high glucose (HG) early induces apoptotic changes in tubular cells and that these changes are prevented by several endogenous and exogenous antioxidants (5). To elucidate further the possible relations between oxidative stress and apoptosis in tubular cells, we studied the time course of changes in intracellular ROS production and apoptosis-related proteins, as well as apoptotic changes in human tubular proximal cells (HK-2 cell line) exposed to hyperglycemia. Our data support the hypothesis that apoptosis induced by hyperglycemia is mediated by ROS and that a downregulation of antiapoptotic X chromosome–linked protein (XIAP) is a major component of this response.

Materials and Methods
HK-2 cells, an immortalized proximal tubular epithelial cell (PTEC) line from normal adult human kidney, were obtained from American Type Culture Collection. Cells were grown to subconfluency and maintained for 18 to 48 h in serum-free medium containing 5.5 (NG) or 30 (HG) mM glucose. In separate experiments, HK-2 were maintained in serum-free medium containing 5.5 mM glucose + 24.5 mM mannitol (HM) (5). To evaluate apoptotic phenomena, we considered the percentage of cells that were annexin V positive/propidium iodide negative (5). For Western blot analysis, cell pellets were treated as described previously (5). Briefly, equal amounts of proteins (40 µg/lane) were diluted with nonreducing sample buffer and separated by electrophoresis on a 12% SDS-PAGE gel. The proteins were electrotransferred to Hybond-C-nitrocellulose membrane. For immunoblot analysis, blots were incubated with primary antibody (anti-XIAP) for 1 h at room temperature on shaking, then
washed in PBS/Tween 20 0.05% (vol/vol), pH 7.4, and, finally, incubated in secondary horseradish peroxidase–labeled antibody for 1 h at room temperature. Bound secondary antibody was detected using enhance chemiluminescence. The intracellular formation of ROS was detected using the fluorescence probe 2′-7′ dichlorofluorescein diacetate. After treatment, HK-2 were treated as described previously (9). To evaluate electrophoretic mobility-shift assay (EMSA) for NF-κB activity, nuclear extracts were prepared from HK-2 cells by using the NE-PER Nuclear and Cytoplasmatic Extraction Kit (Pierce/Celbio, Pero, Italy) according to the manufacturer’s instruction. EMSA was performed using the kb DNA sequence of the Ig gene (5'-CCGGTCAGGGGACTTTC-CGAGACT). Nuclear extracts (2 μg) were incubated with 50 kcpm of 32P-labeled NF-κB oligonucleotide in a binding reaction mixture (10 mM Tris-HCl [pH 7.5], 80 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 1.5 μg of poly [dI-dC]) for 30 min in ice. The DNA-protein complexes were separated on 6% polyacrylamide gels.

All data are presented as means ± SD. Significant difference among data sets was determined by either the unpaired t test or ANOVA with post hoc testing (Statview; Abacus, Berkeley, CA). Differences were considered to be statistically significant at P < 0.05.

Results

Time Course of Intracellular ROS Production and Apoptotic Changes Induced by HG in PTEC

The exposure of HK-2 cells to 30 mM glucose was associated with an early increase in ROS production, which declined thereafter (Figure 1A). ROS production was already increased (by approximately 65%) at 18 h and augmented by 75% after 24 h (P < 0.05 versus NG). At 48 h, ROS production was lower than baseline values. Changes in ROS production at 24 h predicted apoptosis at 48 h (r = 0.96, P < 0.0001). Mannitol 30 mM had no effect on ROS formation.

Exposure of HK-2 cells to 30 mM glucose for 24 h did not result in significant changes in apoptosis. When cells were subjected to HG for 48 h, we observed a significant appearance in early apoptotic changes (34 ± 7% of annexin-positive, propidium-negative cells versus 10 ± 3% for 5.5 mM glucose; P < 0.001; Figure 1B). A similar change in osmolarity obtained by the use of 30 mM mannitol failed to induce apoptotic changes.

Effects of HG on Apoptosis-Related Proteins

HG-mediated apoptosis was preceded at 18 h by a decrease in the expression of antiapoptotic protein XIAP (Figure 2). This event was not observed in HK-2 grown in normal glucose. HG-mediated apoptosis was associated with an increase in the Bax/Bcl2 ratio at 48 h (data not shown; immunostaining and Western Blot analysis). Again, these effects were not reproduced by high osmolality. NF-κB binding activity was markedly reduced when cells were grown for 18 h in HG (Figure 2). The effect of HG was not reproduced by normal glucose.

Discussion

We have observed that the increase in intracellular ROS production during incubation of PTEC with HG media is an early effect, which precedes by several hours the occurrence of apoptotic changes. Lately, when a significant percentage of cells show evidence of apoptosis, intracellular ROS formation declines. According to these findings, ROS generated by accelerated glucose metabolism might represent a signaling molecule for early apoptotic changes. Are the increase in intracellular ROS production and apoptosis related phenomena, or do they represent the concurrence of unrelated effects? The prevention of apoptosis in PTEC by different antioxidants, such as GSH, N-acetylcysteine (NAC), and taurine, as shown recently (5), indicates a cause–effect relationship between ROS and early apoptotic changes. In addition, we observed that the early changes in intracellular ROS formation induced by HG predicted the variations in the apoptotic index. This suggests a close relationship between ROS formation and apoptosis. Moreover, concurrent with the increased ROS formation, the expression of XIAP was blunted by HG medium. XIAP is part of the family of cellular inhibitors of apoptosis, which directly

Figure 1. Effect of glucose (5.5 or 30 mmol/L) on intracellular generation of reactive oxygen species (ROS; A) and apoptosis assessed by annexin V/propidium iodide (B). (A) For studying ROS, cells were treated with normal glucose (NG), high glucose (HG), or high mannitol (HM) for 18, 24, and 48 h and then treated with 5 μmol/L dichlorofluorescein diacetate for 5 min (*P < 0.05 versus NG). (B) Effects of NG, HG, and M on apoptosis. Proximal tubular epithelial cells were treated for 24 to 48 h. (*P < 0.001 versus NG). Data are expressed as means ± SD and are representative of four separate experiments. Every experiment was carried out in duplicate.
bind and inhibit effector caspases, such as caspase-3 and -7, as well as prevent activation of pro-caspase-6 and -9 (10). XIAP can inhibit caspases by preventing cleavage by pro-caspases or by direct inhibition of activated caspases (10). According to these findings, hyperglycemia may exert a permissive effect of apoptosis of PTEC by downregulating the endogenous inhibitors of caspases.

It is suggested that cellular inhibitors of apoptosis are regulated by NF-kB activity (11). In several cell lines, NF-kB has been identified as a target for ROS-dependent signals. Studies about the influence of NF-kB expression on cell survival after oxidants have resulted in conflicting results. In some studies, NF-kB has been suggested to cause a protective effect in response to oxidative stress (12), whereas others support a role of NF-kB as a proapoptotic factor (13). To determine whether NF-kB is influenced by ROS-dependent signals in HK-2 maintained in 30 mM glucose, we performed gel shift assays with nuclear proteins and an NF-kB binding site–specific probe. We observed that when PTEC were grown for 18 h in HG, NF-kB binding activity was switched off. This finding, in addition to those showing a decrease in the expression of NF-kB–induced XIAP, suggests that in this experimental setting, NF-kB has a prosurvival and antiapoptotic function.

In summary, data presented here suggest that ROS are generated as an early signal in human PTEC, which subsequently develop apoptotic changes under HG media, implicating ROS as potential mediators of glucose-induced apoptosis. A downregulation of antiapoptotic protein XIAP seems to be a major component of this response. These findings are consistent with several other observations implicating ROS in the pathogenesis of diabetic complications.

Acknowledgments
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References
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Figure 2. (A) Expression of X chromosome–linked protein (XIAP) was evaluated by Western blot. Cells subjected to HG for 18 h showed the absence of XIAP protein with respect to NG. (B) NF-kB binding to DNA under NG and HG condition in HK-2 cells at 18 h. NF-kB–binding activity is blunted in HG-treated cells.