Hyperglycemia Activates p53 and p53-Regulated Genes Leading to Myocyte Cell Death

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To determine whether enzymatic p53 glycosylation leads to angiotensin II formation followed by p53 phosphorylation, prolonged activation of the renin-angiotensin system, and apoptosis, ventricular myocytes were exposed to levels of glucose mimicking diabetic hyperglycemia. At a high glucose concentration, O-glycosylation of p53 occurred between 10 and 20 min, reached its peak at 1 h, and then decreased with time. Angiotensin II synthesis increased at 45 min and 1 h, resulting in p38 mitogen-activated protein (MAP) kinase–driven p53 phosphorylation at Ser 390. p53 phosphorylation was absent at the early time points, becoming evident at 1 h, and increasing progressively from 3 h to 4 days. Phosphorylated p53 at Ser 18 and activated c-Jun NH2-terminal kinases were identified with hyperglycemia, whereas extracellular signal-regulated kinase was not phosphorylated. Upregulation of p53 was associated with an accumulation of angiotensinogen and AT1, and enhanced production of angiotensin II. Bax quantity also increased. These multiple adaptations paralleled the concentrations of glucose in the medium and the duration of the culture. Myocyte death by apoptosis directly correlated with glucose and angiotensin II levels. Inhibition of O-glycosylation prevented the initial synthesis of angiotensin II, p53, and p38-MAP kinase (MAPK) phosphorylation and apoptosis. AT1 blockade had no influence on O-glycosylation of p53, but it interfered with p53 phosphorylation; losartan also prevented phosphorylation of p38-MAPK by angiotensin II. Inhibition of p38-MAPK mimicked at a more distal level the consequences of losartan. In conclusion, these in vitro results support the notion that hyperglycemia with diabetes promotes myocyte apoptosis mediated by activation of p53 and effector responses involving the local renin-angiotensin system. Diabetes 50:2363–2375, 2001

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A biochemical event with diabetes is the formation of glycosylated products (1). Proteins constitute the principal substrate of this reaction, which generates glycoproteins in the extracellular compartment, plasma membrane, cytoplasm, and, ultimately, in the nucleus (2,3). The most frequent type of intracellular glycosylation is O-linked N-acetylgalactosamine (GlcNAc). This post-translational modification consists of single GlcNAc residues that are connected to the hydroxyl group of serine or threonine by a transferase catalyzing the O-glycosylation (3,4). These sites are clustered at the COOH-terminal of intracellular proteins, in the proximity of proline or valine residue (5). They are similar to phosphorylation sites for several protein kinases (3,6). Glycosylation and phosphorylation activate several transcription factors (7), including p53 (8). The processes of glycosylation and phosphorylation are tightly and dynamically regulated; they affect the activation and stability of the p53 protein (8–10). There is direct competition between glucose and phosphate at a single amino acid residue, resulting in a decrease in the level of phosphorylation when glycosylation occurs, and vice versa (3,6). Because p53 binding sites are present in the promoter of angiotensinogen (Aogen) and AT1 receptor genes (11,12), p53 enhances the myocyte renin-angiotensin system (RAS) and the formation of angiotensin II (Ang II). Moreover, p53 reduces the expression of genes opposing cell death, such as Bcl-2, and upregulates genes promoting apoptosis, such as Bax (13). In this report, the hypothesis was raised that high amounts of glucose lead to enzymatic glycosylation of p53, which activates the myocyte RAS. Synthesis of Ang II and binding to AT1 receptors may trigger distal events upregulating p38 mitogen-activated protein (MAP) kinase (14), which, in turn, may phosphorylate p53 at the COOH-terminal, sustaining its transcriptional activity (15). Additionally, the possibility of phosphorylation of p53 at the NH2-terminal was examined because this site of activation is implicated in several stress-induced cellular responses (16–18). Phosphorylation of c-Jun NH2-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) was also evaluated to recognize the role of these enzymes in glucose-mediated apoptosis (19–21). The decreased Bcl-2–to–Bax protein ratio in myocytes, induced by enhanced p53 function, should depress the resistance of cells to the death signals transmitted by Ang II (11,12,22). Because type 1 diabetes and type 2 diabetes are characterized by elevated plasma glucose concentrations (23),

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Aogen, angiotensinogen; Ang II, angiotensin II; ATF-2, activating transcription factor-2; BAG, benzyl 2-acetamido-2-deoxy-D-galactopyranoside; CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; GlcNAc, N-acetylgalactosamine; HRP, horseradish-peroxidase; JNK, c-Jun NH2-terminal kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; PI, propidium iodide; PMSF, phenylmethanesulfonyl fluoride; RAS, renin-angiotensin system; SFM, serum-free medium; TdT, terminal deoxynucleotidyl transferase; TFA, trifluoroacetic acid; TBST, Tris-buffered saline/Tween 20.
ventricular myocytes were exposed in vitro to hyperglycemia to identify its role in cell death mechanisms.

**RESEARCH DESIGN AND METHODS**

**Cell culture.** Left ventricular myocytes were isolated from male Sprague-Dawley rats at 3 months. Under chloral hydrate anesthesia (300 mg/kg body wt), hearts were excised, and myocytes were dissociated by collagenase (11,24).

Trypan-blue–excluding cells constituted 85%. Nonmyocytes accounted for 1–2%. Myocytes were cultured in serum-free medium (SFM) and in the absence of insulin. After 30 min, the medium was changed with SFM containing 0.5, 1.25, or 25 mmol/l glucose, corresponding to plasma levels of 100, 225, and 450 mg/dl, respectively. Hyperosmolar stress was mimicked by decreasing salts, and acidosis was corrected by adding 20 mmol/l HEPES. Conversely, hyperosmolality was mimicked by including 20 mmol/l mannitol. The effect of the AT1 antagonist losartan (10–7 mol/l), O-glycosylation blocker, benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG, 4 mmol/l; Sigma, St. Louis, MO), and p38-MAPK kinase (MAPK) inhibitor (SB 202190, 1 mmol/l; Calbiochem, San Diego, CA) were determined; these compounds were added to the medium 1 h before glucose. Cultures were studied at 20, 45 min, and 1 h, 3, 12, 24, 48, and 96 h. Myocytes were fixed in 1% formaldehyde for cytometry, and they were frozen at –80°C for biochemistry (11,12,22).

**p53 glycosylation.** Myocytes were suspended in lysate buffer consisting of 50 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA, 250 mmol/l NaCl, 0.1% Triton X-100, dithiothreitol, and 1 mmol/l Na 3VO4. A volume of 50 µg proteins were separated on 12% SDS-PAGE, transferred into nitrocellulose. Carbohydrate moieties were oxidized with 10 mmol/l sodium periodate, followed by incorporation of biotin hydrazide. Biotinylated complexes were detected by streptavidine-alkaline phosphatase and BCIP/NBT color development reagents (Bio-Rad, Hercules, CA). Protein glycosylation was confirmed by performing enzymatic deglycosylation with O-glycosidase D and hexosaminidase, before periodate oxidation (25). Glycosylated p53 was detected by short-term labeling of adherent myocytes with [35S]methionine followed by protein glycosylation at 45 min and 1 h (Fig. 1). Glycosylation of a 55.6-kDa protein (Fig. 1) and 53-kDa band, Bax as a 19-kDa band, Bcl-2 as a 25-kDa band, Aogen as a 56- to 58-kDa band, AT as a 41-kDa band, and AT as a 42- to 44-kDa band (11,12,22).

**Western blot.** Proteins (50 µg) were separated on SDS-PAGE, transferred onto nitrocellulose, and exposed to monoclonal anti-human Mdm2 (SMP14; Santa Cruz Biotechnology), goat polyclonal AT1 (C-18; Santa Cruz Biotechnology) antibodies, and 1 µmol/l TBST. Bound antibodies were detected by HRP-conjugated anti-mouse, anti-rabbit, or anti-goat IgG. p53 was detected as a 53-kDa band, Bax as a 19-kDa band, Bel-2 as a 25-kDa band, Aogen as a 56- to 58-kDa band, AT as a 41-kDa band, and AT as a 42- to 44-kDa band (11,12,22).

**Terminal deoxynucleotidyl transferase assay.** Cell cultures were incubated with 50 µCi cNAME 3H (dissolved in SFM with 25 mmol/l glucose; 5.5 mmol/l glucose was used as a control. With high glucose, osmolarity was adjusted to 290 mOsm. Glycoprotein detection by periodate oxidation showed glycosylation of a 55.6-kDa protein (Fig. 1A and B). The increase in protein glycosylation seen at 10 min with high glucose was not statistically significant. However, a threefold increase in optical density at 20 min (0.50 ± 0.20 vs. 1.7 ± 0.5, P < 0.001) and a sixfold increase at 45 min (0.42 ± 0.20 vs. 2.6 ± 0.8, P < 0.001) was demonstrated; glycosylation peaked sevenfold (0.44 ± 0.12 vs. 3.1 ± 0.7, P < 0.001) at 1 h and remained elevated fourfold (0.56 ± 0.20 vs. 2.2 ± 0.5, P < 0.001) at 3 h. A progressive decrease in glycosylation of the 55.6-kDa protein was noted at 12, 24, 48, and 96 h. Enzymatic deglycosylation before periodate oxidation prevented the appearance of the 55.6-kDa glycopeptide (Fig. 1A and B).

**RESULTS**

**p53 glycosylation.** To evaluate whether p53 became glycosylated, myocytes were cultured in SFM at 25 mmol/l glucose; 5.5 mmol/l glucose was used as a control. With high glucose, osmolarity was adjusted to 290 mOsm. Glycoprotein detection by periodate oxidation showed glycosylation of a 55.6-kDa protein (Fig. 1A and B). The increase in protein glycosylation seen at 10 min with high glucose was not statistically significant. However, a threefold increase in optical density at 20 min (0.50 ± 0.20 vs. 1.7 ± 0.5, P < 0.001) and a sixfold increase at 45 min (0.42 ± 0.20 vs. 2.6 ± 0.8, P < 0.001) was demonstrated; glycosylation peaked sevenfold (0.44 ± 0.12 vs. 3.1 ± 0.7, P < 0.001) at 1 h and remained elevated fourfold (0.56 ± 0.20 vs. 2.2 ± 0.5, P < 0.001) at 3 h. A progressive decrease in glycosylation of the 55.6-kDa protein was noted at 12, 24, 48, and 96 h. Enzymatic deglycosylation before periodate oxidation prevented the appearance of the 55.6-kDa glycopeptide (Fig. 1A and B).

**In the presence of high glucose, losartan did not interfere with protein glycosylation at 45 min and 1 h (Fig. 1C). Conversely, inhibition of O-glycosylation by BAG mark-
edly attenuated the 55.6-kDa protein (Fig. 1D). The 55.6 kDa glycoprotein was identified as p53 by radiolabeling myocytes exposed to 5.5 and 25 mmol/l glucose with [35S]methionine for 1, 2, and 3 h. Myocyte lysates were then immunoprecipitated with pAb 240 anti-p53 (Fig. 1E). At high glucose, two proteins at 55.6 and 53 kDa were detected. The lower–molecular weight band corresponded to p53, and the higher molecular weight band reflected the addition of carbohydrates to the p53 protein.

To identify the sugar moiety bound to p53, myocyte lysates obtained from cells cultured in the presence of 5.5 and 25 mmol/l glucose were immunoprecipitated with pAb 240 anti-p53. Immunoprecipitated proteins were subjected to Western blot with the monoclonal antibody RL2, which recognized O-GlcNAc linked to p53 (Fig. 1F). The O-glycosylated form of p53 was apparent at 55.6 kDa. O-GlcNAc-p53 was detected in myocytes exposed to high glucose for 20 min (0.8 ± 0.2, n = 5; P < 0.001), peaking at 1 h (4 ± 0.5, n = 5; P < 0.001). The level of O-GlcNAc-p53 remained stable at 3 h (3.5 ± 0.7, n = 5; P < 0.001) and decreased progressively at 12 h (1.6 ± 0.4, n = 5; P < 0.001) and 24 h (1.2 ± 0.3, n = 5; P < 0.001).

p53 glycosylation and Ang II formation. To establish whether O-glycosylation of p53 upregulated the myocyte RAS, Ang II concentration, expressed as picograms per hour per 10^6 cells, was measured by ELISA in CM collected from myocytes cultured at 5.5 and 25 mmol/l glucose under isosmotic conditions. In comparison with 5.5 mmol/l glucose, Ang II formation at 25 mmol/l glucose did not increase at 10 and 20 min (values were combined), but increased 1.4-fold at 45 min, 1.6-fold at 1 and 3 h (values were combined), and 1.8-fold at 12 h (Fig. 2). Inhibition of O-glycosylation by BAG prevented the increase in Ang II at each time point.

p53 phosphorylation at the COOH-terminus. The next question concerned whether p53 glycosylation–mediated Ang II formation resulted in p53 phosphorylation. Phosphorylation of p53 at 55.6 kDa was examined in IMs cultured at 5.5 and 25 mmol/l glucose, exposed to p53 antibodies recognizing, respectively, the site where phosphorylation by PKC at Ser 376 occurred and the site where phosphorylation by p38-MAPK at Ser 390 occurred. p53 phosphorylation at Ser 376 was undetectable at 5.5 mmol/l glucose, nor was it detectable at 25 mmol/l glucose after 10, 20, and 45 min (Fig. 3A and B). With high glucose, p53 phosphorylation was clearly visible at 1 h (2.4 ± 0.7, n = 5). A progressive increase in phosphorylated p53 at Ser 376 was noted at 3 h (3.6 ± 0.9, n = 5; P < 0.05), 12 h (4.5 ± 1.1, n = 5; P < 0.01), 24 h (6.4 ± 1.8, n = 5; P < 0.001).
Activation of p38-MAPK by Ang II. Ang II concentration

**FIG. 2.** Ang II generation. Ang II levels in CM of myocytes exposed to low and high glucose are shown at 10–20 min, 45 min, 1–3 h, and 12 h. BAG prevents the increase in Ang II formation at 25 mmol/l glucose at all time points. Results are the means ± SD. ∗P < 0.05 vs. 5.5 mmol/l glucose and vs. 5.5 and 25 mmol/l glucose at 10–20 min; †P < 0.05 vs. 25 mmol/l glucose at the same time point. Values of n vary from 7 to 17.

0.005), 48 h (11 ± 3, n = 5; P < 0.001), and 96 h (15 ± 4, n = 5; P < 0.001). Losartan and BAG prevented phosphorylation (Fig. 3C and D).

At 25 mmol/l glucose, there was an increase with time, from 1 h to 4 days, in the level of nonactivated p53 at Ser 376 that corresponds to the site of p53 activation by PKC (Fig. 3E). The increase in the latent form of p53 with high glucose followed the decrease in p53 glycosylation, because pAb 421 anti-p53 does not recognize glycosylated p53. Glycosylated p53 peaked at 1 h, when nonactivated p53 at Ser 376 was not detectable. As expected, the amount of nonactivated p53 at 5.5 mmol/l glucose was similar to that at 25 mmol/l, reflecting the lack of p53 phosphorylation at Ser 376 with low and high glucose concentrations. Losartan did not modify the level of nonactivated p53 at Ser 376 (Fig. 3F), indicating that translocation of PKC by Ang II was not involved in p53 phosphorylation. Optical density values for nonactivated p53 are not shown.

**p53 phosphorylation at the NH2-terminus.** Phosphorylated p53 at Ser 18 represents an additional site of activation of the tumor suppressor in response to DNA damage (16–18). Thus, phosphorylated p53 at Ser 18 was evaluated. Phosphorylated p53 was not detectable at 5.5 mmol/l glucose. At 25 mmol/l glucose, phosphorylated p53 at Ser 18 was not apparent at 45 min, but its quantity increased progressively at 1 h (0.11 ± 0.02, n = 5), 3 h (0.37 ± 0.09, n = 5; P < 0.05), 12 h (0.52 ± 0.11, n = 5; P < 0.001), 24 h (0.69 ± 0.12, n = 5; P < 0.001), 48 h (0.91 ± 0.13, n = 5; P < 0.001), and 96 h (1.36 ± 0.25, n = 5; P < 0.001) (Fig. 3G).

**Phosphorylated p53 at Ser 390 and Ang II formation.** Phosphorylation of p53 at the COOH-terminal and at Ser 390 is mediated by the activation of p38-MAPK (15). To strengthen the notion that p53 phosphorylation by this MAPK followed p53 glycosylation–induced Ang II formation, myocytes were cultured at 5.5 and 25 mmol/l glucose in the presence of an inhibitor of p38-MAPK (SB 202190) or an AT1 antagonist (losartan). AT1 blockade prevents activation of p38-MAPK by Ang II. Ang II concentration was then measured under two conditions of suppressed p38-MAPK. Values at 10 and 20 min and at 1 and 3 h were comparable and, thus, combined. When phosphorylation was blocked, the production of Ang II was not decreased for up to 3 h but was inhibited at 12 h (Fig. 3F). At 12 h, p53 glycosylation was significantly decreased (Fig. 1A–C), whereas p53 phosphorylation at Ser 390 was upregulated (Fig. 3A–C). Losartan produced effects similar to SB 202190. The attenuated synthesis of Ang II at 12 h by SB 202190 and losartan documented that p53 phosphorylation was a secondary event triggered by p53 glycosylation.

**p38-MAPK activity.** To confirm that p38-MAPK was stimulated by Ang II and was responsible for p53 phosphorylation at Ser 390, phosphorylated p38 was measured by Western blot in myocytes cultured at 5.5 and 25 mmol/l glucose in isosmotic media (Fig. 4A and B). Phosphorylated p38 was undetectable at all time points with 5.5 mmol/l glucose; this was also the case at 25 mmol/l glucose after 10, 20, and 45 min. At 25 mmol/l glucose, phosphorylated p38 became apparent at 1 h (0.5 ± 0.2, n = 5) and 3 h (0.7 ± 0.3, n = 5). Phosphorylated p38 progressively increased at 12 h (1.0 ± 0.3, n = 5; P < 0.005), 24 h (1.5 ± 0.5, n = 5; P < 0.003), 48 h (2.1 ± 0.7, n = 5; P < 0.002), and 96 h (2.8 ± 0.9, n = 5; P < 0.001). p38-MAPK enzymatic activity was also assessed by measuring ATF-2 phosphorylation. ATF-2 is a physiological substrate of p38-MAPK. Phosphorylated ATF-2 was not visible in myocytes at 5.5 mmol/l glucose. However, in cells treated with 25 mmol/l glucose, activated ATF-2 increased from 1 to 96 h (Fig. 4C; optical density values not shown). Earlier intervals were not examined because phosphorylated p38 was not present. We thus established that activation of p38-MAPK was caused by Ang II synthesis induced by p53 glycosylation. Western blots were performed with myocytes treated with the inhibitor of O-glycosylation, BAG, 1 h before the exposure of the cells to low and high glucose concentrations. The active form of the enzyme was undetectable in these samples (Fig. 4D).

**ERK and JNK kinases.** To complete the analysis of the effects of glucose on the MAPK family of proteins, the active forms of ERK and JNK were examined. Phosphorylated ERK1 and -2 levels were not detected in myocytes cultured in the presence of low and high glucose (data not shown). In contrast, the active forms of JNK1 and JNK2 were recognized at high glucose (Fig. 4E). This was apparent at 20 min (0.61 ± 0.08, n = 5) and consistently increased at 1 h (1.1 ± 0.11, n = 5; P < 0.05), 3 h (1.27 ± 0.22, n = 5; P < 0.01), 12 h (2.38 ± 0.31, n = 5; P < 0.001), 24 h (2.67 ± 0.34, n = 5; P < 0.001), and 48 h (2.9 ± 0.4, n = 5; P < 0.001). Phosphorylated JNK was not detected at 5.5 mmol/l glucose.

**p53 DNA binding.** To document that p53 phosphorylation enhanced p53 function, gel mobility assays were performed. In comparison with myocytes at 5.5 mmol/l glucose, the optical density of the p53-shifted band for the Aogen promoter increased 5-fold (P < 0.001) and 12-fold (P < 0.001) in cells exposed to 25 mmol/l glucose for 1 day (0.42 ± 0.13 vs. 2.2 ± 0.6) and 2 days (0.45 ± 0.21 vs. 5.3 ± 1.9), respectively (Fig. 5A). Osmolarity did not affect p53 binding. Controls included preincubation of nuclear extracts with a p53 antibody or with an excess of unlabeled self-oligonucleotide; both conditions prevented the forma-
tion of a p53-shifted complex. Conversely, the addition of an irrelevant antibody did not interfere with the p53 band.

With respect to cells at 5.5 mmol/l glucose, myocytes cultured at 25 mmol/l glucose showed a fourfold \( (P < 0.001) \) and an eightfold \( (P < 0.001) \) increase in the p53-shifted complex for the AT1 promoter at one \((0.13 \pm 0.05 \text{ vs. } 0.53 \pm 0.18)\) and two \((0.18 \pm 0.10 \text{ vs. } 1.4 \pm 0.33)\) d, respectively (Fig. 5B). Similarly, high glucose increased p53 DNA binding to the Bax promoter fourfold \( (P < 0.001) \) and ninefold \( (P < 0.001) \) at one \((1.1 \pm 0.4 \text{ vs. } 4.2 \pm 1.2)\) and two \((1.3 \pm 0.9 \text{ vs. } 12 \pm 4)\) d (Fig. 5C). Consistency in protein loading, lack of protein degradation, and uniformity in the relative purity of nuclear extracts were evaluated by SDS-PAGE and Coomassie blue staining (not shown).

**Western blot.** The expression of p53, Bax, Bcl-2, Aogen, AT1, and AT2 receptors was evaluated in myocytes exposed to 5.5, 12.5, and 25 mmol/l glucose for 4 days. We have previously shown that conditions characterized by hyperglycemia-induced Ang II formation \((30–32)\), or enhanced Ang II synthesis alone, independent from hyperglycemia \((11,12,22)\), led to upregulation of p53 and p53-associated genes. Currently, p53 protein increased in proportion to glucose concentration \((0.23 \pm 0.07, 1.0 \pm 0.22, \text{ and } 2.2 \pm 0.6 \text{ for } 5.5, 12.5, \text{ and } 25 \text{ mmol/l, respectively, } n = 6 \text{ in all cases}; P < 0.05–0.001 \text{ vs. } 5.5 \text{ mmol/l})\) and losartan, at \(10^{-7} \text{ mol/l,} \) completely abolished the effect of hyperglycemia on p53 quantity (data not shown). p53 activation was paralleled by comparable increases in Bax \((2.3 \pm 0.5, 7.9 \pm 1.7, \text{ and } 13 \pm 2.3 \text{ for } 5.5, 12.5, \text{ and } 25 \text{ mmol/l, respectively, } n = 6 \text{ in all cases}; P < 0.001 \text{ vs. } 5.5 \text{ mmol/l})\), whereas an inverse correlation was observed between glucose and Bcl-2 expression \((9.3 \pm 1.6, 6.6 \pm 1.0, \text{ and } 3.7 \pm 0.7 \text{ for } 5.5, 12.5, \text{ and } 25 \text{ mmol/l, respectively, } n = 6 \text{ in all cases}; P < 0.001 \text{ vs. } 5.5 \text{ mmol/l})\); losartan prevented the changes in Bax and Bcl-2. Aogen \((0.73 \pm 0.18, 2.4 \pm 0.4, \text{ and } 3.8 \pm 0.7 \text{ for } 5.5, 12.5, \text{ and } 25 \text{ mmol/l, respectively, } n = 6 \text{ in all cases}; P < 0.005–0.001 \text{ vs. } 5.5 \text{ mmol/l})\); losartan prevented the changes in Bax and Bcl-2.
showing higher levels with higher glucose concentrations. As expected, AT2 receptors were not modified by glucose (3.7 ± 0.5, 4.0 ± 0.7, and 3.6 ± 0.5 for 5.5, 12.5, and 25 mmol/l, respectively, n = 6 in all cases; NS) or losartan. Hyperosmolarity did not significantly change any of the listed values.

**Ang II.** To demonstrate that enhanced p53 function and upregulation of Aogen with hyperglycemia resulted in a long-term increase in Ang II synthesis, Ang II was measured in CM after exposure of myocytes to 5.5, 12.5, and 25 mmol/l glucose.

**FIG. 4.** p38-MAPK and JNK activation. A and B: At 25 mmol/l glucose (Glu), p38-MAPK is phosphorylated at threonine 180 and tyrosine 182 (phospho-p38 MAPK) at 1 h, and its expression increases progressively with time. Anisomycin-treated NIH-3T3 cells are used as positive control (+), whereas untreated NIH-3T3 cells are used as negative control (−). C: The enzymatic activity of phosphorylated p38-MAPK is assessed by the phosphorylation of ATF-2 at threonine 71 (phosphorylated ATF-2). D: BAG prevents phosphorylation of p38-MAPK at 25 mmol/l glucose. E: The active forms of JNK1 and -2 are detectable with high glucose at 20 min and consistently increase with time. Phosphorylated JNK is not seen at 5.5 mmol/l glucose.

**FIG. 5.** p53 DNA binding activity. A, B, and C: At 25 mmol/l glucose (Glu), p53 phosphorylation enhances p53 binding to the promoter for Aogen, AT1, and Bax. Osmolarity has no effect on p53 binding. I, isosmotic medium; H, hyperosmotic medium. Preincubation of nuclear extracts with a p53 antibody (Ab) or with an excess of unlabeled self oligonucleotide (Co) prevents the formation of a p53-shifted complex. Conversely, the addition of an irrelevant antibody (Irr) does not interfere with the p53 band. Ao, AT1, and Bax: angiotensinogen, AT1, and Bax probes in the absence of nuclear extracts. Arrows point to the p53-shifted complex.
mmol/l glucose for 1, 2, and 4 days (Fig. 6). Osmolarity did not affect Ang II levels, and values were combined. In comparison with 5.5 mmol/l glucose, 25 mmol/l glucose increased Ang II formation 1.9-fold at 1 day. Additionally, Ang II generation increased 1.9- and 2.5-fold at 2 days and 2.6- and 3.9-fold at 4 days with 12.5 and 25 mmol/l glucose, respectively.

**Myocyte apoptosis.** This form of cell death is characterized first by nuclear damage, which is frequently not detectable morphologically. However, double DNA strand breaks can be identified histochemically. This initial phase of the apoptotic process is followed by nuclear fragmentation and later by cytoplasmic alterations (33). To determine the extent of the apoptotic process is followed by nuclear fragmentation and later by cytoplasmic alterations (33). To determine whether Ang II activated cell death, myocyte apoptosis was measured. Figure 7A–I illustrates by confocal microscopy three myocytes undergoing apoptosis. Nuclear morphology was preserved in Fig. 7A–C, whereas nuclear fragmentation was apparent in Fig. 7D–I. Cell striation was evident in the first example. Loss of cytoplasmic structure and cell shrinkage were seen in the other two cases.

Apoptosis was measured in myocytes cultured at 5.5, 12.5, and 25 mmol/l glucose for periods of 1, 2, and 4 days. Cell death increased with time and glucose concentration (Fig. 7J). Hyperosmolarity of the medium did not alter the degree of apoptosis at any time point or glucose concentration, including 4 days and 25 mmol/l glucose. Values collected under isosmotic and hyperosmotic conditions were combined. The lack of impact of increased osmolarity on apoptosis was confirmed by measuring cell death at 5.5 mmol/l glucose in the presence of 20 mmol/l mannitol for 4 days. Osmolarity increased to a value comparable to that found at 5.5 mmol/l glucose, with 2.8 ± 1.2 and 3.6 ± 1.7% for control and mannitol, respectively (n = 6; P = 0.6). At 4 days of hyperglycemia, apoptosis comprised nearly 3, 8, and 15% of myocytes at 5.5, 12.5, and 25 mmol/l glucose, respectively. Moreover, to evaluate the impact of BAG on myocyte apoptosis, 4 mmol/l of this inhibitor of O-glycosylation was added to myocytes cultured at 25 mmol/l glucose in isosmotic medium. Myocyte apoptosis was measured 4 days later, with 2.5 ± 1.1% for 5.5 mmol/l glucose (n = 6), 13 ± 4% for 25 mmol/l glucose and no BAG (n = 6; P < 0.001), and 3.5 ± 1.7% for 25 mmol/l glucose plus BAG (n = 6; P = 0.7). Thus, hyperosmolarity had no effect on the magnitude of myocyte apoptosis, and BAG blocked high glucose–mediated cell death.

**Apoptosis, losartan, and pH.** To examine the effects of AT₁ blockade on cell death, losartan was added 1 h before glucose and maintained for up to 4 days. Losartan attenuated apoptosis by nearly 60% in all conditions but failed to decrease cell death to baseline (Fig. 7K). These values of apoptosis were obtained by TdT. Enhanced uptake of glucose by myocytes leads to the generation of lactic acid, thereby decreasing extracellular pH (34). The effects of glucose concentration and time on the pH of the medium are shown in Fig. 7L. Essentially, 5.5 mmol/l glucose did not decrease pH during the 4-day period. However, 25 mmol/l glucose reduced pH throughout the duration of the culture. To prevent the development of acidosis, 20 mmol/l HEPES were added to the medium (Fig. 7L). The influence of pH on hyperglycemia-induced apoptosis was determined. The impact of losartan on cell death was also re-examined under low and physiological pH. TdT detects double-strand DNA cleavage induced by Ca²⁺-dependent DNase I and pH-dependent DNase II (11, 29), but it cannot separate the effects of these two DNases. Conversely, the hairpin probe identifies double-strand DNA cleavage with a single-base 3′ overhang, which is the form of apoptosis mediated by Ang II exclusively via the stimulation of Ca²⁺-dependent DNase I (11, 29).

Figure 7M illustrates the extent of myocyte apoptosis in cell cultures at 5.5 and 25 mmol/l glucose in the presence and absence of losartan for 4 days. These experiments were performed in isosmotic media. At 25 mmol/l glucose and pH 6.9, myocyte apoptosis measured by the hairpin probe was significantly lower than that assessed by TdT. As expected, losartan did not abolish cell death in the presence of acidosis, resulting in a significant TdT labeling of myocyte nuclei. In contrast, at 25 mmol/l glucose and pH 7.4, apoptosis evaluated by hairpin probe was almost identical to that determined by TdT. As expected, losartan reduced cell death to control values when measured by either TdT or hairpin probe. Thus, losartan completely inhibited myocyte apoptosis triggered by Ang II formation with hyperglycemia.

**Acidosis, phosphorylated p53 at Ser 390, and phosphorylated p38 MAPK.** In view of the presence of acidosis with hyperglycemia, we evaluated the potential effects of low pH on the main pathways implicated in the activation of p53 and p38-mediated apoptosis in this model system. The levels of phosphorylated p53 at Ser 390 and phosphorylated p38 MAPK were not affected by pH in myocytes cultured for 2 and 4 days at 25 mmol/l glucose. The presence or absence of HEPES did not change the activity of these two proteins (Fig. 8A and B). These observations confirmed that the biochemical results obtained with high glucose concentration were not influenced by pH.

**DISCUSSION**

The findings of the present study indicate that high glucose concentrations led to O-glycosylation of p53 and synthesis of Ang II in adult cardiac myocytes. Binding to AT₁ receptors promoted the activation of p38-MAPK, which in turn induced p53 phosphorylation at Ser 390 in the COOH ter-
minus. This post-translational modification sustained p53 function and the upregulation of p53-dependent and -regulated genes. p53 stability and transcriptional activity were also influenced by its phosphorylation at Ser 18 in the NH₂ terminus. Conversely, hyperglycemia induced a dual phosphorylation of JNK at Thr 183 and Tyr 185 (19,21), most likely facilitating the degradation of p53 (35,36). However, these multiple responses enhanced p53 activity,
increasing the susceptibility of myocytes to undergo Ang II–mediated apoptosis. The extent of myocyte death directly correlated with glucose and Ang II levels. With hyperglycemia, intracellular acidosis became apparent, and this metabolic defect accounted for nearly 40% of the myocyte death. The AT₁ blocker losartan prevented the effects of Ang II on cell death but had no influence on apoptosis triggered by acidosis. Importantly, inhibition of O-glycosylation counteracted this cascade of events by interfering with the initiation of p53 function and the
FIG. 7—Continued.
generation of Ang II. Thus, hyperglycemia upregulated the myocyte RAS, which is implicated in the development of a diabetic cardiomyopathy in vivo (30–32).

**Hyperglycemia and p53 activation.** Glycosylation of proteins with hyperglycemia in vivo and in vitro occurs through the addition of carbohydrates, without involving an enzymatic reaction (1,2). This process is commonly defined as glycation or nonenzymatic glycosylation. However, the formation of glycation products requires time and is not evident before cells are kept in high glucose-containing media for at least 1 week (37). The rate of glycation is so slow that rapidly turning-over intracellular proteins would not be present long enough to undergo nonenzymatic glycosylation (38). This is relevant to p53, which has a half-life of 20 min (10). The enzymatic O-glycosylation of p53 occurred between 10 and 45 min and progressively decreased after 3 h. The addition of O-GlcNAc to the COOH-terminal of p53 was replaced by phosphorylation, which became apparent only after 1 h of hyperglycemia. Phosphorylated p53 increased consistently from 3 h to 4 days. Inhibition of O-glycosylation prevented p53 phosphorylation at Ser 390, p38 MAPK activation, Ang II synthesis, and apoptosis. Thus, O-glycosylation and phosphorylation were sequentially implicated in the modulation of p53 function and transactivation of p53-dependent and -regulated genes in myocytes. Hyperglycemia-mediated O-glycosylation of p53 has never been shown before. Similarly, enzymatic glycosylation of p53 has never been documented in normal cells. The only example was obtained in a carcinoma-derived cell line in which wild-type glycosylated p53 is constitutively expressed and is biologically active (8). Glycosylated p53 promotes transcription of target genes and induces apoptosis.

Glycosylation and phosphorylation activate several transcription factors (7,8), ~20 of which, including SP1, AP-1, SRF, and p53, are known to be O-glycosylated (8). The basic region at the COOH-terminus of p53 represses DNA binding, maintaining p53 in its latent form (9). This inhibition can be relieved by various mechanisms: introduction of bulky groups, including sugars like GlcNAc (9), or neutralization of charge through phosphorylation by PKC, casein kinase II, and p38-MAPK (9,10). The sites of glycosylation do not possess a common consensus sequence, but some shared characteristics exist (5). These sites are clustered at the COOH-terminus in the proximity of proline and valine residues. They are located in sequences rich in serine and threonine (5) that are similar to phosphorylation sites for protein kinases (4–6). These sites constitute the substrate for the catalytic activity of glycosyl transferase and protein kinases (6). The role of the hexosamine pathway in p53 glycosylation has been demonstrated here by the identification of O-GlcNAc residues bound to the transcription factor.

Phosphorylation of p53 was examined at three distinct sites: two at the COOH-terminus and one at the NH2 terminus. In the first two cases, phosphorylation of the tumor suppressor was restricted to Ser 390 and was mediated by p38-MAPK. In contrast, there was no apparent p53 phosphorylation at Ser 376, indicating the lack of involvement of PKC in the activation of p53 with hyperglycemia. This conclusion was based on the reactivity detected with antibody 421, which recognizes p53 when phosphate groups (9,27,28,39) do not occupy the epitope 372–381. At the NH2-terminus, p53 was phosphorylated at Ser 18, a site that is critical for p53 function in response to DNA damage (17). After DNA injury, phosphorylation at the NH2-terminus of p53 is relevant to both the stabilization of this protein and its role in apoptosis and growth arrest (18). Cell death and block of the cell cycle in G1 depend on p53 conformational changes associated with phosphorylation of Ser residues distributed at the NH2 terminus (18). Ser 6, 9, 15, 20, and 46 are selectively phosphorylated by different kinases activated by DNA-damaging agents (16–18,40). DNA injury promotes a consistent phosphorylation of p53 at Ser 15 in humans and at its homolog in rodents, Ser 18 (17,18,41). We elected to evaluate phosphorylated p53 at Ser 18 for its critical role in the initiation of cell death as a result of DNA damage. Our findings on myocyte apoptosis and double DNA strand breaks with hyperglycemia are consistent with this possibility.

**Hyperglycemia and MAPK.** p38-MAPK, JNK, and ERK were investigated to identify their effects on hyperglycemia-mediated responses in myocytes. Downstream events triggered by ligand binding to AT1 receptor were involved in phosphorylation of p38-MAPK (14). This signaling pathway was confirmed by the use of the AT1 antagonist losartan. Treatment of myocytes with the AT1 blocker had no influence on p53 O-glycosylation, but it abolished p53 phosphorylation. Losartan also prevented p38-MAPK activation. Inhibition of p38-MAPK by SB 202190 mimicked, at a more distal level, the consequences of losartan by preventing phosphorylated p53 and thus the synthesis and secretion of Ang II. The three MAPK families react differently to hormonal and metabolic stimuli, and the variability in their functional characteristics reflects the cell type involved (19–21). As shown above, hyperglycemia stimulates p38-MAPK and JNK in myocytes in a manner similar to vascular endothelial cells (19,21,42,43) and smooth muscle cells (44,45). Conversely, ERK is not activated by high glucose concentrations in these cell populations, but it is upregulated in adipocytes and fibroblasts (20). Ang II activates JNK through the AT1 receptor in vascular smooth muscle cells and it inhibits ERK through the AT2 receptor (45). Whether AT1 receptors have a similar effect on myocytes is currently unknown.

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**FIG. 8. pH and phosphorylation of p53 and p38-MAPK.** A and B: At high glucose (Gl), pH does not influence the levels of phosphorylated (phospho) p53 at Ser 390 and phosphorylated p38-MAPK at 2 and 4 days. Acidosis was corrected by 4 mmol/l HEPEs (HE).
Because of the primary role played by p38-MAPK in myocytes exposed to hyperglycemia, we chose to selectively block this enzyme instead of using a more general inhibitor of MAPK, such as a MEK inhibitor. The latter would have affected the proapoptotic action of p38-MAPK and the antiapoptotic function of JNK (14,15,35,36), complicating the interpretation of the accumulated results.

**Hyperglycemia and p53-mediated RAS activation.** Diabetes is characterized by upregulation of RAS (30,32), and interventions decreasing Ang II attenuate morbidity and mortality of diabetic patients (46,47). Inhibition of ACE positively modifies cardiovascular events and nephropathy in subjects with severe diabetes (47). However, the mechanism by which diabetes upregulates the systemic and/or local RAS remains to be identified. Ventricular myocytes possess a cellular RAS and synthesize and secrete Ang II (11). Additionally, Ang II stimulates cell death and cellular hypertrophy (11,24). The possibility that diabetes enhances the local RAS, resulting in the development of cardiac myopathy, has been shown in vivo (30,32); myocyte apoptosis and myocyte hypertrophy have been identified in the diabetic heart. AT1 blockade prevents these phenomena as well as the induction of p53-regulated genes interfering with cell death and cell growth.

p53 has been linked to the transactivation of Aogen and AT1 receptor, leading to the formation and release of Ang II from myocytes (11,12,22). p53 binding to the promoter of Aogen and AT1 increased with high glucose concentrations, and this adaptation resulted in the accumulation of Aogen and AT1 receptor proteins in myocytes. Aogen is the limiting factor in the generation of Ang II in these cells (22). Inhibition of p53 downregulates the expression of Aogen, attenuating the myocyte RAS and the synthesis of Ang II (12,22). In this study, glucose levels, p53 expression, and Ang II quantity have been found to be directly correlated. The increased Ang II in the medium 45 min after hyperglycemia most likely reflects de novo synthesis and release of Ang II from myocytes. This notion is consistent with the short half-life of the octapeptide, which is 6–12 min in the cells and 15 s in the blood (48,49). In the current experiments, the formation of Ang II followed the O-glycosylation of p53. Therefore, it is reasonable to assume that p53-mediated activation of the local RAS stimulated the generation and secretion of the peptide from the stressed myocytes.

**Hyperglycemia and Ang II-mediated apoptosis.** Ligands binding to surface AT1 receptors trigger myocyte death by a mechanism involving an increase in cytosolic Ca2+ and the activation of Ca2+-dependent DNase I (11,22). pH-dependent DNase II is not implicated in Ang II-mediated apoptosis (11,50). The present results are consistent with these observations, emphasizing the importance of hyperglycemia in enhancing the synthesis of Ang II and AT1 receptor, which constitute the death signal and the executor of myocyte apoptosis, respectively. AT1 and AT2 receptors have distinct roles in the initiation of apoptosis in various cell types. In PC12W and R3T3 cells, which lack AT1 receptors, Ang II stimulation of AT2 results in internucleosomal DNA cleavage through dephosphorylation of the survival factor MAPK (51,52). In vascular smooth muscle cells, AT2 receptor activation opposes ERK function, promoting apoptosis (53). AT1 receptors induce cell survival by interfering with the effects of AT2 on ERK activity (54). The claim has been made that neonatal and adult cardiac myocytes behave in a comparable manner in the presence of Ang II: AT2 triggers apoptosis, and AT1 inhibits cell death (55,56). The discrepancy between these results and our observations is difficult to explain. However, culture systems were not comparable, and much higher doses of Ang II, losartan, and PD123319 were used in these other experiments. These factors may account for the difference.

In conclusion, these vitro results, in combination with in vivo observations (30–32), support the notion that hyperglycemia is responsible for apoptotic myocyte death with diabetes via activation of effector responses involving the local RAS. Importantly, a strong association between the upregulation of cellular RAS and the apoptosis of myocytes, endothelial cells, and fibroblasts has been identified in the diabetic human heart (31). Our work in diabetic patients (31) and experimental animals (30,32) is the only one that has emphasized the role of cell dropout in the initiation and progression of the diabetic myopathy. Because of the critical role of hyperglycemia in cell death, the current findings in vitro may apply to both juvenile type 1 diabetes and adult type 2 diabetes.

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