Loss of Heparan N-Sulfotransferase in Diabetic Liver
Role of Angiotensin II
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The basis for accelerated atherosclerosis in diabetes is unclear. Diabetes is associated with loss of heparan sulfate (HS) from the liver, which may impede lipoprotein clearance and thereby worsen atherosclerosis. To study hepatic HS loss in diabetes, we examined regulation of HS N-deacetylase/N-sulfotransferase-1 (NDST), a key enzyme in hepatic HS biosynthesis. Hepatic NDST mRNA, protein, and enzymatic activity were suppressed by >50% 2 weeks after induction of type 1 diabetes in rats. Treatment of diabetic rats with enalapril, an ACE inhibitor, had no effect on hyperglycemia or hepatic NDST mRNA levels, yet increased hepatic NDST protein and enzymatic activity. Similar results were obtained in diabetic animals treated with losartan, which blocks the type 1 receptor for angiotensin II (AngII). Consistent with these findings, diabetic livers exhibited increased ACE expression, and addition of AngII to cultured hepatoma cells reduced NDST activity and protein. We conclude that diabetes substantially suppresses hepatic NDST mRNA, protein, and enzymatic activity. AngII contributes to suppression of NDST protein and enzymatic activity, whereas mRNA suppression occurs independently. Suppression of hepatic NDST may contribute to diabetic dyslipidemia, and stimulation of NDST activity by AngII inhibitors may provide cardiovascular protection. Diabetes 54:1116–1122, 2005

Diabetes is associated with an increased risk for atherosclerotic cardiovascular disease, the major cause of death in this population, but the basis for increased atherosclerosis is unclear (1). A major contributing factor is diabetic dyslipidemia (1–3). Normal uptake and catabolism of atherogenic lipoproteins requires heparan sulfate (HS) in the liver (rev. in 4–8). Diabetes is associated with substantially reduced hepatic HS sulfation in vivo (9) and with impaired hepatic uptake of lipoproteins (10,11). Hepatic HS may be especially important in the clearance of postprandial lipoproteins (10–12), which accumulate in human diabetic plasma (2,3) and may be particularly atherogenic (13,14).

Thus, hepatic HS biosynthesis is a crucial issue in diabetes. The content of HS and extent of HS sulfation in the liver are strongly influenced by the HS N-deacetylase/N-sulfotransferase-1 (NDST). This critical enzyme catalyzes N-deacetylation and N-sulfation of HS, and this N-sulfation indirectly stimulates HS chain elongation (15,16), epimerization (17,18), and O-sulfation (17,18). Impairment of N-deacetylase activity has been reported in diabetic hepatocytes (19) and liver (20). Nevertheless, molecular regulation of NDST, and in particular its role in the impairment of HS sulfation in diabetic liver, has yet to be described in vivo.

Several regulatory factors relevant to HS sulfation have been examined in vitro. Cultured hepatocytes showed only minimal reductions (~15%) in HS sulfation when incubated in high concentrations of glucose (10). Likewise, high glucose concentrations had no effect on NDST mRNA or HS N-sulfation in cultured adipocytes (21) or fibroblasts (22). A 50% suppression in NDST mRNA was reported in vascular smooth muscle cells exposed to high-glucose medium (23), suggesting differential regulation in vitro depending on cell type. The findings in cultured hepatocytes, however, suggest that hyperglycemia itself may not be the key signal to cause the large suppression of hepatic HS sulfation in vivo. In addition to high glucose levels, angiotensin II (AngII) has been closely linked to diabetic complications. Prolonged exposure of cultured mesangial cells to AngII was reported to suppress HS synthesis and N-sulfation (24), although no studies have looked at AngII effects on HS in liver cells. AngII may be particularly important in the accelerated atherosclerosis of diabetes, based on clinical observations that ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) may provide cardioprotection to diabetic patients beyond blood pressure reduction (25,26).

In this study, we sought to evaluate the effects of diabetes in vivo on the molecular control of hepatic NDST as well as a possible role for AngII. We found a marked suppression of NDST mRNA, protein, and N-sulfotransferase activity in livers of streptozotocin (STZ)-induced diabetic rats. Importantly, we also found that inhibition of AngII generation or blockade of AngII receptors substan-
tially restored NDST protein and activity in diabetic livers. Moreover, ACE mRNA levels were increased in diabetic liver, consistent with increased local AngII action, and supplementation of liver cells in vitro with AngII likewise suppressed NDST activity and protein.

RESEARCH DESIGN AND METHODS
Male Sprague-Dawley rats (7–8 weeks of age, 200–250 g; Harlan-Teklad, Wilkerson, WI) were injected intraperitoneally with 65 mg/kg body wt STZ in sodium citrate buffer (pH 4.5) to induce type 1 diabetes (27). Normal control animals received vehicle only. Animals with plasma glucose concentrations >300 mg/dl 3 days after STZ injection were considered diabetic. Low-dose insulin was administered to prevent severe weight loss and ketosis. Blood glucose levels were assayed at 7 and 14 days to verify persistent diabetes, and then the animals were killed. Samples of liver were snap-frozen in liquid nitrogen for subsequent determinations of sulfotransferase activities, NDST mRNA levels, and NDST protein by Western blotting. Some samples were fixed in buffered formalin and then embedded in paraffin for immunohistochemical analysis. Enalapril (50 mg/kg drinking water), an ACEI, or losartan (10 mg/kg drinking water), an ARB that blocks the type 1 receptor for AngII, was administered to subsets of diabetic animals from days 3 to 14. All animal studies were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Cell culture experiments. McArdle 7777 rat hepatoma cells were seeded on plates coated with poly-D-lysine and grown in medium with 10% FCS and 10% horse serum, as previously described (28). To examine the effects of AngII, cells were plated in 36-mm wells (Co-Star/Corning, Cambridge, MA) at about 50% confluence. After an overnight incubation, cells were switched to medium with 2% serum for 24 h and then supplemented with 0, 1.0, or 50 μmol/L AngII. Cells were harvested 48 h later for sulfotransferase activity assays, Western blotting, and RNA analyses.

Sulfotransferase activities. Assays of organ samples for sulfotransferase activities were performed by a modification of previously published methods (28). Snap-frozen samples of rat liver were homogenized in 0.25 mol/l sucrose and 50 mmol/L Tris-HCl (pH 7.5) with protease inhibitors (1 μmol leupeptin, 0.5 μg/ml pepstatin, and 0.2 mmol/l phenylmethylsulfonyl fluoride) by a high-speed rotating blade (Polytron) followed by sonication. Monolayers of McArdle cells were scraped into the same sucrose buffer and homogenized. To initiate the HS-sulfotransferase reaction, 36 μl of each sample of homogenized organ or cells was mixed with 2.0 mol/l [35S]phosphosulfate (PAPS) (Perkin-Elmer Life Sciences, Boston, MA), the sulfate acceptor. Both PAPS and phosphatase (PAPS) (Perkin-Elmer Life Sciences, Boston, MA), the sulfate acceptor. Both PAPS and phosphatase (PAPS) (Perkin-Elmer Life Sciences, Boston, MA), the sulfate acceptor. Both PAPS and phosphatase (PAPS). We validated these assays by observing slopes of approximately 3.32 when cycle threshold (Ct) was plotted against the log10 of template cDNA dilution, which indicates efficient PCR, and an essentially horizontal curve when the difference in Ct values between NDST and PPIA (ΔCt) was plotted against the log10 of template cDNA dilution, which indicates independence of this parameter from input template mass. Levels of NDST mRNA are expressed as a percentage of the PPIA mRNA levels (100 × 2−ΔCt). Assays of ACE mRNA were performed similarly, using the sequences 5′-aatcagcggaatggcgaggt-3′ (sense primer), 5′-ccaggctttccttcgg-3′ (antisense primer), and 5′-ctctaggtcaattccc-3′ (probe). Sequences for the PPIA reactions were 5′-cttcggctgctgctcc-3′ (sense primer), 5′-ctctcctccggctgta-3′ (antisense primer), and 5′-aaggagattcccttt3′ (probe).

Statistical analyses. Unless otherwise indicated, summary statistics are reported as means ± SE. Comparisons of several groups simultaneously were performed by initially using ANOVA. When the ANOVA indicated significant differences among the groups, pairwise comparisons were performed using the Student-Newman-Keuls q statistic (34).

RESULTS
We began by comparing hepatic NDST in normal control rats, diabetic rats, and diabetic rats treated with enalapril. Average blood glucose levels in the three groups at the end of the study were 92.5 ± 3.3, 410 ± 59, and 572 ± 25 mg/dl, respectively (means ± SE, n = 4 per group). Figure 1A shows representative TLC spots from the HIS N-sulfotransferase assay of liver homogenates, indicating suppression in diabetes and partial recovery with an ACEI. Chondroitin sulfotransferase spots indicate no change with any treatment (Fig. 1B). Figure 1C shows quantitative data: the filled columns indicate a significant 63% suppression of NDST enzymatic activity in the livers of diabetic rats. The addition of ACEIs to diabetic rats produced a significant recovery of NDST activity despite continued hyperglycemia. As a control, the open columns in Fig. 1C indicate that diabetes and an ACEI had no effect on hepatic chondroitin sulfotransferase activity.

We next determined whether these alterations in NDST enzymatic activity originated at the level of protein or mRNA. Western blotting indicated substantial suppression of NDST protein in diabetic liver, with substantial restoration upon ACEI treatment (Fig. 2A). By immunostaining,
hepatic parenchymal cells from nondiabetic animals uniformly exhibited a strong NDST signal, with a widespread granular pattern within each cell (Fig. 2B). Diabetes provoked a substantial suppression of NDST protein staining in liver, with many hepatic parenchymal cells exhibiting no detectable staining at all (Fig. 2C). There was also a redistribution of NDST immunostaining to a peripheral staining pattern in cells that still maintained residual NDST staining in the diabetic livers. Importantly, livers from diabetic animals treated with an ACEI showed an intermediate pattern, with a greater percentage of cells showing NDST immunostaining, and a restoration of the widespread granular staining pattern within each cell that was stained (Fig. 2D). These immunohistochemical findings are displayed quantitatively in Table 1. Therefore, diabetes suppresses and an ACEI partially restores hepatic NDST enzymatic activity, protein mass, and protein distribution. Surprisingly, the NDST mRNA levels showed a different pattern. By real-time PCR, diabetes produced a significant 50% suppression of hepatic NDST mRNA compared with saline-treated controls, but an ACEI failed to restore it (Fig. 3A). These results indicate an overall increase in AngII action in diabetic livers. To explore the mechanism, we examined hepatic levels of ACE mRNA and found a significant increase with diabetes (Fig. 3B).

Similar results were obtained in a separate study comparing normal rats, diabetic rats, and diabetic rats treated with losartan, an ARB. Diabetes caused a significant 54% suppression of NDST enzymatic activity, and the addition
of an ARB produced a significant 61% increase over diabetes without drug (Fig. 4A, filled columns). Again, chondroitin sulfotransferase activity was indistinguishable among the treatment groups (Fig. 4A, open columns). Western blotting showed suppression of NDST protein with diabetes and restoration with an ARB (Fig. 4B). Nevertheless, similar to our ACEI study, diabetes significantly suppressed NDST mRNA by 56%, but an ARB did not restore it (Fig. 5). As before, diabetes more than doubled hepatic ACE mRNA levels (not shown).

Finally, to examine a causal link between AngII and NDST regulation, we incubated McArdle rat hepatoma cells for 48 h in vitro with different concentrations of this hormone. Consistent with our findings in vivo, we found significant suppression of NDST enzymatic activity by AngII (Fig. 6A), as well as suppression of NDST protein (Fig. 6B), but without a decrease in NDST mRNA (Fig. 6C).

### Table 1

<table>
<thead>
<tr>
<th>Zone</th>
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<td>W</td>
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<td>48.3 ± 3.8*</td>
<td>P</td>
<td>DM + ACEI</td>
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<td>DM + ACEI</td>
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Data are means ± SE. Zone 1 refers to the area around portal triads; zone 2 is the intermediate region; and zone 3 is around central veins. NL, normal rats; DM, diabetic rats; DM + ACEI, diabetic rats treated with enalapril; W, widespread staining within each cell; P, staining limited to the periphery of each cell. For each zone, P < 0.01 by ANOVA for the comparison of the percentage of stained cells among the three treatment groups. *P < 0.01 compared with NL; †P < 0.01 compared with DM by the Student-Newman-Keuls test.

![FIG. 3. Effects of diabetes and ACE inhibition on hepatic NDST (A) and ACE (B) mRNA levels. RNA was extracted from samples of livers from the same experiment as in Figs. 1 and 2, followed by mRNA quantitation by real-time PCR. In each panel, mRNA levels are expressed as a percentage of the level of PPIA mRNA (P < 0.05 by ANOVA for each panel; *P < 0.05 compared with normal, by the Student-Newman-Keuls test; n = 5–6).](image)

![FIG. 4. Effects of diabetes and angiotensin receptor blockade on hepatic sulfotransferase activities and NDST protein content. The experimental design was identical to the one in Fig. 1, except that losartan was used instead of enalapril. A: Hepatic N-sulfotransferase activity (■; P < 0.001 by ANOVA; *P < 0.05 compared with normal; †P < 0.05 compared with diabetic, by the Student-Newman-Keuls test; n = 3–5) and total chondroitin sulfotransferase activity (□; NS). B: NDST Western blot. Lanes 1 and 2, normal; lanes 3 and 4, diabetic; lanes 5 and 6, diabetic + enalapril. The arrow indicates the single NDST protein band.)](image)
DISCUSSION

We found that diabetes suppresses hepatic levels of mRNA, protein, and enzymatic activity for NDST, a key regulatory enzyme in HS biosynthesis. The diabetic effect on NDST mRNA levels appears to occur independently of AngII. Nevertheless, we found that AngII is a major contributor to NDST protein and enzymatic suppression in liver cells both in vivo and in vitro. These results suggest that some protective effects of renin-angiotensin blockade in diabetes may involve restoration of hepatic NDST.

The role of HS in physiology is often overlooked, owing to the existence of proteins that serve related, though not identical, functions. In the liver, lipoprotein uptake has been shown to occur via members of the LDL receptor family, particularly the LDL receptor itself (35) and the LDL receptor–related protein (36). Nevertheless, HS proteoglycans in the liver, such as syndecan and perlecain, are also able to directly mediate endocytosis of lipoproteins and other ligands in vitro (4–8,37–39). Moreover, there is evidence that HS proteoglycans play a direct role in vivo in hepatic catabolism of lipoproteins, particularly the large, postprandial particles (12). Importantly, a major component of the dyslipidemia of diabetes is postprandial (2,3), and there is evidence that it results, at least in part, from loss of hepatic HS (9,10,19,20). Our current results indicate that a key enzyme in hepatic HS synthesis, NDST-1, is significantly suppressed at the mRNA and protein levels in diabetic livers. This process is likely to contribute to proatherogenic diabetic dyslipidemia via decreased hepatic HS sulfation and consequent impairment of lipoprotein clearance.

Based on our current findings, one might expect that blockage of AngII would ameliorate diabetic dyslipidemia by increasing the expression and function of NDST in the diabetic liver. These drugs, however, have generally been regarded as “lipid neutral” (rev. in 40,41), but this conclusion has been based on studies that are almost entirely limited to examinations of fasting plasma and mostly in nondiabetic subjects. One study of the effect of ACEIs on postprandial fat tolerance was performed in hypertensive patients with impaired glucose tolerance (42). The study showed that ACEIs did improve postprandial fat tolerance, consistent with our current findings, but the subjects’ glucose tolerance also improved (42), which complicates the interpretation. A more recent brief report found a cholesterol-lowering effect of ARB in patients with type 1 diabetes and albuminuria (43), which would also be con-

FIG. 5. Effects of diabetes and angiotensin receptor blockade on hepatic NDST mRNA levels. RNA was extracted from samples of livers from the same experiment as in Fig. 4, followed by mRNA quantitation by real-time PCR. Displayed are NDST mRNA levels expressed as a percentage of the level of PPIA mRNA ($P < 0.01$ by ANOVA; $^* P < 0.05$ compared with normal; $n = 5$).

FIG. 6. Effect of AngII on sulfotransferase activities and NDST mRNA in liver cells in vitro. McArdle 7777 hepatoma cells were cultured for 48 h with 0.0 (control), 1.0, or 50 μmol/l Ang II, as indicated. A: HS N-sulfo transferase activities ($P = 0.01$ by ANOVA; $^* P < 0.05$ compared with control; $n = 3$). Chondroitin sulfo transferase activities were undetectable (not shown). B: Representative NDST Western blots, with the NDST band indicated by the arrow. C: NDST mRNA levels normalized to cyclophilin mRNA (NS by ANOVA; $n = 3$).
sistent with our current findings. Thus, it is possible that improvements in plasma lipid levels, particularly in the postprandial state, might contribute to cardiovascular protection from these medicines in diabetes.

There is now a widespread consensus that the renin-angiotensin system is critical in mediating local tissue damage in diabetes and contributes greatly to overall diabetic complications. The most compelling evidence is the remarkable reduction in cardiovascular morbidity and mortality in diabetic patients treated with an ACEI (25) or an ARB (26). Unfortunately, methodologic and other problems have prevented a clear demonstration that any specific, pathogenic component of the renin-angiotensin system is actually stimulated in diabetes. In patients with diabetes, the plasma renin levels are low. Pro-renin levels are elevated and correlate with diabetic complications (44), but the function of pro-renin is unclear. Systemic ACE and AngII levels in diabetic patients are not elevated, although subsets of patients may have elevated ACE activity if they carry the ACE DD polymorphism (45,46). Another possibility is that diabetes stimulates local increases of AngII or AngII receptors within specific tissues or compartments (47). It is now accepted that most tissues contain all of the components of the renin-angiotensin system and are able to generate tissue AngII independent of the circulation. Regarding the liver, hepatic parenchymal cells were previously shown to respond to AngII and to contain type I receptors for AngII (48), but data regarding effects of diabetes on the renin-angiotensin system within the liver are essentially nonexistent. In this study, we provide evidence to support a causal chain: diabetes increases ACE expression in the liver, which should enhance local AngII action (Fig. 3B); addition of AngII to cultured liver cells suppresses NDST activity and protein (Fig. 6); and blockade of AngII production (Figs. 1 and 2) significantly suppresses NDST, a key molecule in hepatic HS biosynthesis. AngII contributes to the decrease in liver NDST protein and enzymatic activity, but independent from effects on NDST mRNA. These results suggest post-transcriptional regulation of NDST, which has been demonstrated in vitro in nonhepatic cells (49).

Overall, we conclude that early diabetes in vivo significantly suppresses NDST, a key molecule in hepatic HS biosynthesis. AngII contributes to the decrease in liver NDST protein and enzymatic activity. The ability of AngII blockade to favorably modify the expression and function of this enzyme in diabetes could substantially improve postprandial lipoprotein clearance and other HS-dependent functions.

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Portions of this work were presented at the American Heart Association Scientific Sessions, Anaheim, California, 12 November 2001 (50).

REFERENCES

4. Williams KJ, Fless GM, Petrie KA, Snyder ML, Obunike JC, Naggi A, Casu B, Goldberg IJ: Enhanced local AngII action (Fig. 3) increases ACE expression in the liver, which should enhance effects of diabetes on the renin-angiotensin system (47). It is now accepted that most tissues contain all of the components of the renin-angiotensin system and are actually stimulated in diabetes. In patients with diabetes, the plasma renin levels are low. Pro-renin levels are elevated and correlate with diabetic complications (44), but the function of pro-renin is unclear. Systemic ACE and AngII levels in diabetic patients are not elevated, although subsets of patients may have elevated ACE activity if they carry the ACE DD polymorphism (45,46). Another possibility is that diabetes stimulates local increases of AngII or AngII receptors within specific tissues or compartments (47). It is now accepted that most tissues contain all of the components of the renin-angiotensin system and are able to generate tissue AngII independent of the circulation. Regarding the liver, hepatic parenchymal cells were previously shown to respond to AngII and to contain type I receptors for AngII (48), but data regarding effects of diabetes on the renin-angiotensin system within the liver are essentially nonexistent. In this study, we provide evidence to support a causal chain: diabetes increases ACE expression in the liver, which should enhance local AngII action (Fig. 3B); addition of AngII to cultured liver cells suppresses NDST activity and protein (Fig. 6); and blockade of AngII production (Figs. 1 and 2) significantly suppresses NDST, a key molecule in hepatic HS biosynthesis. AngII contributes to the decrease in liver NDST protein and enzymatic activity, but independent from effects on NDST mRNA. These results suggest post-transcriptional regulation of NDST, which has been demonstrated in vitro in nonhepatic cells (49).

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