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Research Article

Although supraphysiological concentrations of urea are known to increase oxidative stress in cultured cells, it is generally thought that the elevated levels of urea in chronic renal failure patients have negligible toxicity. We previously demonstrated that ROS increase intracellular protein modification by O-linked β-N-acetylglucosamine (O-GlcNAc), and others showed that increased modification of insulin signaling molecules by O-GlcNAc reduces insulin signal transduction. Because both oxidative stress and insulin resistance have been observed in patients with end-stage renal disease, we sought to determine the role of urea in these phenotypes. Treatment of 3T3-L1 adipocytes with urea at disease-relevant concentrations induced ROS production, caused insulin resistance, increased expression of adipokines retinol binding protein 4 (RBP4) and resistin, and increased O-GlcNAc–modified insulin signaling molecules. Investigation of a mouse model of surgically induced renal failure (uremic mice) revealed increased ROS production, modification of insulin signaling molecules by O-GlcNAc, and increased expression of RBP4 and resistin in visceral adipose tissue. Uremic mice also displayed insulin resistance […]

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Urea-induced ROS generation causes insulin resistance in mice with chronic renal failure

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Although supraphysiological concentrations of urea are known to increase oxidative stress in cultured cells, it is generally thought that the elevated levels of urea in chronic renal failure patients have negligible toxicity. We previously demonstrated that ROS increase intracellular protein modification by O-linked β-N-acetylglucosamine (O-GlcNAc), and others showed that increased modification of insulin signaling molecules by O-GlcNAc reduces insulin signal transduction. Because both oxidative stress and insulin resistance have been observed in patients with end-stage renal disease, we sought to determine the role of urea in these phenotypes. Treatment of 3T3-L1 adipocytes with urea at disease-relevant concentrations induced ROS production, caused insulin resistance, increased expression of adipokines retinol binding protein 4 (RBP4) and resistin, and increased O-GlcNAc–modified insulin signaling molecules. Investigation of a mouse model of surgically induced renal failure (uremic mice) revealed increased ROS production, modification of insulin signaling molecules by O-GlcNAc, and increased expression of RBP4 and resistin in visceral adipose tissue. Uremic mice also displayed insulin resistance and glucose intolerance, and treatment with an antioxidant SOD/catalase mimetic normalized these defects. The SOD/catalase mimetic treatment also prevented the development of insulin resistance in normal mice after urea infusion. These data suggest that therapeutic targeting of urea-induced ROS may help reduce the high morbidity and mortality caused by end-stage renal disease.

Introduction
Elevated urea in chronic renal failure (CRF) is considered to have negligible toxicity because Johnson et al. found in 1972 that in patients with far-advanced renal failure, blood urea concentrations of less than 50 mM (blood urea nitrogen [BUN] of 140 mg/dl) were well tolerated when blood urea concentration was maintained by adding urea to the dialysate solution (1) and 30 years later, the HEMO study showed that increasing urea reduction rate from 66% to 75% did not alter survival in patients with an increased dialysis dose (2). However, more recent data have demonstrated that survival of patients on daily hemodialysis is 2- to 3-fold greater than that of patients dialyzed less frequently (3). The observation that in an animal model of CRF-accelerated atherosclerosis (4), plasma urea was the only significant predictor of aortic plaque area fraction suggested to us that the high levels of urea found in chronic dialysis patients might play an important role in accelerated atherosclerosis in this group of patients.

Nonphysiologic concentrations of urea have been reported to increase levels of ROS and the oxidative stress marker 8-oxoguanine in cultured IMCD3 cells (5). End-stage renal disease (ESRD) patients have increased levels of oxidative stress (6), and we have shown previously that intracellular ROS induced by hyperglycemia increase protein modification by O-linked β-N-acetylglucosamine (O-GlcNAc) (7). Increased modification of key signaling molecules by O-GlcNAc was recently shown to cause reduced insulin signal transduction (8). Insulin resistance is a well-documented feature of ESRD, and the rate of death among hemodialysis patients is greater in those with more severe insulin resistance (9). The insulin resistance present in ESRD appears to start much earlier in the course of chronic kidney disease, when renal failure is subclinical (10). Although the cause of insulin resistance in chronic renal disease is unknown (11), several abnormalities associated with ESRD might interfere with insulin signaling (12–17). We therefore hypothesized that concentrations of urea associated with CRF might drive insulin resistance by increasing ROS production, thereby increasing modification of insulin signaling molecules by O-GlcNAc. In this study, we have observed that uremic mice are insulin resistant and glucose intolerant, with increased plasma levels of 2 insulin resistance–associated adipokines: retinol binding protein 4 (RBP4) and resistin. In cultured 3T3-L1 adipocytes, urea caused decreased insulin sensitivity and increased adipokine secretion due to ROS-induced increases in O-GlcNAc modification of insulin signaling molecules. In uremic mice, treatment with a SOD/catalase mimetic normalized both O-GlcNAc modification of insulin signaling molecules and the increased insulin resistance–associated adipokine expression in visceral adipose tissue, correcting systemic insulin resistance and glucose intolerance. Finally, urea infusion in normal animals induced the same degree of insulin resistance and elevated insulin resistance–associated adipokines seen in uremic mice, while concomitant SOD/catalase mimetic treatment completely prevented insulin resistance and elevated adipokines induced by urea infusion.

Results
Uremic mice are insulin resistant and glucose intolerant and have elevated plasma levels of insulin resistance–associated adipokines. Body weight and food intake did not differ between the uremic and nonuremic
mice throughout the study. In CRF mice, the serum plasma urea concentration was increased 2.8-fold 2 weeks after 5/6 nephrectomy (Table 1).Remarkably, in uremic mice, the fasting plasma insulin levels were more than 6 times higher than those of non-uremic mice, while the plasma glucose concentration trended toward an increase but was not significantly different between the two groups (Table 1), suggesting the presence of insulin resistance. Consistent with an insulin-resistant phenotype (18), plasma triglyceride and cholesterol levels were both significantly increased in the uremic mice (Table 1). To quantify the degree of insulin resistance and glucose intolerance induced by uremia, i.p. insulin and glucose tolerance tests were performed (Figure 1). As shown in Figure 1A, the decrease in plasma glucose 60 minutes after insulin injection was half that in nonuremic mice. Similarly, in uremic mice, elevated plasma glucose remained unchanged 120 minutes after glucose injection (Figure 1B). In control mice, glucose values returned to baseline by 120 minutes while in uremic mice, glucose values remained more than twice baseline values (Figure 1D). Uremic mice had plasma insulin levels 6.4-fold higher than control mice at 0 minutes and 8.6-fold higher than control mice 120 minutes after glucose injection (Figure 1C). Plasma insulin levels correlated closely with plasma urea levels ($r^2 = 0.8; P < 0.01$). Since elevated plasma levels of the insulin resistance–associated adipokines RBP4 and resistin are observed in CRF patients (19, 20), concentrations of RBP4 and resistin were measured in the plasma of uremic CRF and control mice. In uremic mice, plasma RBP4 concentration was increased 2.8-fold (Figure 2A) and plasma resistin levels were 1.7-fold higher compared with those of control mice (Figure 2B).

**Urea reduces insulin-stimulated glucose transport in 3T3-L1 adipocytes by altering phosphorylation of insulin signaling molecules.** In order to determine whether elevated urea was the cause of insulin resistance in uremic mice, insulin-stimulated uptake of [1-3H] 2-deoxyglucose was measured in differentiated 3T3-L1 cells. A concentration-response relationship between urea and impaired insulin signaling was observed between 10 mM and 40 mM urea (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI37672DS1). Remarkably, after incubation with a urea concentration similar to that in uremic mice (20 mM) for 48 hours, insulin-stimulated glucose transport was reduced by 76.4%, whereas mannitol, used as an osmotic control, had no effect on insulin-stimulated glucose transport (Figure 3A). To investigate the mechanism underlying this urea-induced inhibition of insulin-stimulated glucose transport, cell lysates were prepared from adipocytes exposed to insulin alone and to insulin plus 20 mM urea. IRS-1 was immunoprecipitated and blotted for total phosphotyrosine, which activates insulin signaling, and for IRS-1

<table>
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<tr>
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<th>C57BL/6J</th>
<th>Uremic C57BL/6J</th>
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<td>26.4 ± 1.7</td>
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<td>Plasma insulin (pg/ml)</td>
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<td>1680 ± 4.34</td>
<td>&lt;0.01</td>
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<td>Plasma glucose (mg/dl)</td>
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<td>122.66 ± 20.597</td>
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<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>64.27 ± 2.4</td>
<td>166.38 ± 18.05</td>
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<tr>
<td>Plasma triglycerides (mg/dl)</td>
<td>53.51 ± 4.7</td>
<td>102.63 ± 10.28</td>
<td>&lt; 0.01</td>
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Results are expressed as mean ± SEM; n = 34.

**Figure 1**

Uremic mice are insulin resistant and glucose intolerant. (A) Insulin tolerance test. Mice were injected i.p. with insulin (0.75 U/kg), and blood glucose was measured at 0, 30, and 60 minutes. (B) i.p. glucose tolerance test. Mice were injected i.p. with glucose (1 mg/g body weight), and blood glucose was measured at 0, 15, 30, 60, and 120 minutes. (C) Insulin resistance in uremic mice. Insulin values from the IPGTT animals shown in B were determined by ELISA. (D) Glucose intolerance in uremic mice. Glucose values from B at 0 and 120 minutes. All results represent mean ± SEM of 9 animals per group. *$P < 0.01$ compared with controls.
Ser636 phosphorylation, which functionally inhibits IRS-1 activity (8). As shown in Figure 3B, urea reduced insulin-dependent IRS-1 tyrosine phosphorylation by 60%, while it increased Ser636 phosphorylation by 70%. Akt was also immunoprecipitated and blotted for pSer473 (Figure 3C). As with IRS-1, urea reduced insulin-dependent phosphorylation of Ser473 by 46%. Urea treatment did not impair the ability of insulin to stimulate insulin receptor autophosphorylation (data not shown).

Urea-induced decrease in insulin-stimulated glucose transport is mediated by mitochondrial ROS. Nonphysiologic concentrations of urea have been shown to increase free radical generation in renal inner medullary cells (5). We found that urea at a concentration similar to that in uremic mice (20 mM) increases ROS levels in 3T3-L1 adipocytes by 2.9-fold, and insulin had no effect on this urea-induced ROS generation (Figure 4A and Supplemental Figure 1A). Since increased ROS levels appear to increase insulin resistance in several physiologic contexts (21), we evaluated the effect of overexpressing various antioxidant enzymes on the urea-induced decrease in insulin-stimulated glucose transport (Figure 4B). Catalase, a heme protein located predominantly in peroxisomes and the inner mitochondrial membrane, catalyzes the conversion of H₂O₂ to H₂O. Overexpression of catalase normalized both urea-induced ROS generation (Figure 4A) and insulin-stimulated glucose transport (Figure 4B). This suggests that overproduction of H₂O₂ plays a critical role in urea-induced insulin resistance. Since increased H₂O₂ production could reflect functional alterations in mitochondria, endoplasmic reticulum, or a number of NADPH-dependent oxidases, we next overexpressed 2 enzymes that specifically prevent mitochondrial ROS production: uncoupling protein-1 (UCP1), which collapses the proton electrochemical gradient driving mitochondrial ROS production, and manganese SOD2 (MnSOD2), the mitochondrial form of this antioxidant enzyme. Each of these 2 enzymes normalized urea-induced free radical generation (Figure 4A) and completely prevented urea-induced insulin resistance in 3T3-L1 adipocytes (Figure 4B). Together, these data indicate that mitochondrial superoxide overproduction is responsible for the observed insulin resistance in 3T3-L1 adipocytes.

Urea-induced ROS modify IRS-1 by O-GlcNAc. Increased mitochondrial superoxide production has been shown to activate the hexosamine pathway, which results in increased modification of intracellular proteins by O-GlcNAc. Recently, it has been reported that modification of the insulin signaling pathway by O-GlcNAc results in altered phosphorylation of key signaling molecules that attenuated insulin signal transduction (8). As shown in Figure 5A,
Urea treatment doubled IRS-1 modification by O-GlcNAc. This increase was completely prevented by overexpression of catalase, UCP1, or MnSOD (Figure 5B).

**Urea-induced ROS increase expression and secretion of insulin resistance–associated adipokines.** As shown in Figure 2, plasma levels of RBP4 and resistin were increased in uremic mice. In 3T3-L1 adipocytes, urea exposure increased RBP4 expression by 2.5-fold and resistin expression by 2-fold (Figure 6A). Levels of RBP4 and resistin secreted into the medium by cells exposed to urea were 1.7-fold and 3-fold higher, respectively, than those from cells exposed to insulin alone (Figure 6B). Overexpression of catalase, MnSOD, or UCP1 completely prevented the urea-induced increase in expres-

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**Figure 4**
Urea-induced decrease in insulin-stimulated glucose transport is mediated by mitochondrial ROS. Cells were infected with adenoviral vectors expressing catalase, UCP1, or MnSOD. After 48 hours incubation with urea, both intracellular ROS generation (A) and insulin-stimulated glucose uptake (B) were measured. Each bar represents the mean ± SEM of 5 separate experiments, each with n = 8. *P < 0.01 compared respectively with cells not treated with urea (A) or with insulin (B).

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**Figure 5**
Urea-induced ROS increase IRS-1 modification by O-GlcNAc in 3T3L1 adipocytes. (A) IP Western blot analysis of IRS-1 modification by O-GlcNAc in urea-treated 3T3L1 cells (48 hours) and their controls. (B) IP Western blot analysis of IRS-1 modification by O-GlcNAc in urea-treated 3T3-L1 cells alone and after overexpression of catalase, UCP1, and MnSOD. Cells were infected with adenoviral vectors expressing catalase, UCP1, or MnSOD. After incubation with urea for 48 hours, IRS-1 was immunoprecipitated and the amount of O-GlcNAc modification was analyzed by immunoblotting. Levels of modification in cells infected with empty vector are shown as 100% in bar graphs. n = 5; *P < 0.01 compared with controls. Data represent mean ± SEM.
sion of RBP4 (Figure 6C) and resistin (Figure 6D). These results confirm that urea-induced ROS, which cause adipocyte insulin resistance, directly increase expression and secretion of the insulin resistance–associated adipokines RBP4 and resistin from 3T3-L1 adipocytes in a cell-autonomous manner.

**Treatment of uremic mice with a SOD/catalase mimetic normalizes oxidative stress, IRS-1 O-GlcNAc modification, and IRS-1 tyrosine phosphorylation.** Since urea-induced ROS caused insulin resistance in 3T3-L1 adipocytes by increasing IRS-1 modification by O-GlcNAc and reducing IRS-1 tyrosine phosphorylation, we examined these parameters in adipose tissue of uremic mice. In epididymal fat, the level of oxidative stress was determined by measuring protein nitration. As shown in Figure 9A, the level of protein nitration was increased over 6-fold in adipose tissue from uremic mice. Treatment of uremic mice with MnTBAP completely normalized adipose tissue protein nitration. IRS-1 modification by O-GlcNAc treated with MnTBAP was not statistically different from the concentration measured in sham-operated mice (262 ± 29.63 pg/ml versus 265 ± 29.7 pg/ml) (Figure 7C).

This normalization of insulin levels in MnTBAP-treated uremic mice was associated with normalization of both insulin resistance and abnormal glucose tolerance (Figure 8). Treatment of uremic mice with MnTBAP completely normalized their insulin tolerance tests (Figure 8A). Similarly, glucose tolerance in uremic mice was completely normalized by MnTBAP treatment (Figure 8, B and D). In these mice, serum insulin levels were normalized at both 0 minutes and 120 minutes after glucose injection (Figure 8C), and serum glucose was normalized at 120 minutes (Figure 8D).

**Figure 6**

Urea-induced ROS increase expression and secretion of insulin resistance–associated adipokines in 3T3L1 adipocytes. (A) Effect of urea on RBP4 and resistin mRNA. Adipokine expression was evaluated by quantitative PCR (qPCR) in cells exposed to urea for 48 hours and in control cells. (B) Effect of urea on RBP4 and resistin protein secretion in the medium by 3T3L1 adipocytes. RBP4 and resistin concentration were measured by ELISA. (C and D) Effect of catalase, MnSOD, and UCP1 overexpression on urea-induced increases in RBP4 (C) and resistin (D) expression. Cells were infected with adenoviral vectors as described above and adipokine expression measured by qPCR. Results are expressed as fold changes versus the controls. Each bar represents the mean ± SEM of 5 different experiments. *P < 0.01 compared with controls.
Urea infusion in normal animals induces insulin resistance and elevates insulin resistance–associated adipokines. The data presented in Figures 8–10 demonstrate that partially nephrectomized mice exhibit high plasma urea and insulin resistance, which is completely reversed by MnTBAP treatment. To demonstrate that elevated urea actually causes insulin resistance in vivo, urea was infused in normal animals and insulin resistance was determined during euglycemic hyperinsulinemic (EU) clamps (23). Urea was infused for 48 hours prior to the EU clamp, and the steady-state urea concentration in urea-infused animals was 2.9-fold higher than in isotonic saline-infused animals (data not shown), similar to the 2.8-fold increase in urea concentration observed in our CRF model (Table 1). Insulin levels at time zero of the clamps was nearly 2-fold elevated in the uremic animals (0.08 versus 0.14 μg/ml) but was identical (2.1 versus 2.2 μg/ml) during the EU clamp. The glucose infusion rate (GIR) necessary to maintain euglycemia was significantly decreased in the urea-infused animals (Figure 11, A and B), indicating marked whole-body insulin resistance (24). Treatment with MnTBAP completely reversed the urea-induced insulin resistance, with the GIR necessary to maintain euglycemia not statistically different from that of saline-infused animals. Urea-induced insulin resistance was associated with a greater than 2.5-fold increase in plasma levels of the insulin resistance–associated adipokines RBP4 (Figure 11C) and resistin (Figure 11D), similar to the fold increase observed in uremic mice (Figure 2). Moreover, MnTBAP treatment also prevented the urea-induced elevation of blood levels of the insulin resistance–associated adipokines RBP4 (Figure 11C) and resistin (Figure 11D). The urea infusion conditions sufficient to induce insulin resistance did not induce hemolysis or osmotic diuresis, 2 potentially confounding variables. These data demonstrate that urea alone is sufficient to induce the same degree of insulin resistance and elevated insulin resistance–associated adipokines seen in uremic mice and that insulin resistance and elevated serum adipokines in the urea-infused normal rats are caused by urea-induced ROS.

**Discussion**

In nonuremic people with normal glucose tolerance, insulin resistance markedly increases cardiovascular disease risk, even after adjustment for known risk factors such as LDL, triglycerides, HDL, and systolic blood pressure (25, 26). In dialysis patients, the cardiovascular mortality rate is approximately 30 times the risk in the general population and remains 10 to 20 times higher even after stratification for age, sex, and presence of diabetes (27). Over-
all, the 5-year mortality rate for patients on dialysis is 60% (28). CRF itself appears to be a significant contributing cause of this accelerated atherosclerosis, since even young, nondiabetic adults on chronic hemodialysis have increased intimamedia thickening, arterial stiffness, and coronary artery calcification (29–31). The rate of death among hemodialysis patients is higher in those with more severe insulin resistance (9).

Due to the lack of correlation with symptoms of the uremic syndrome after addition of urea to dialysate (1) and the lack of impact on survival in patients whose urea reduction rate was increased from 66% to 75% by increased dialysis dose (2), it has been assumed that elevated levels of urea in CRF have negligible toxicity. However, more recent data show that survival of patients on daily hemodialysis is 2- to 3-fold greater than that of patients dialyzed less frequently (3). Reduction in several insulin resistance metabolic markers was associated with improved mortality in patients on daily hemodialysis, including hyperhomocysteinemia, dyslipidemia, hypoalbuminemia, and hormonal abnormalities (3). ESRD patients also have increased levels of oxidative stress (6), and reduction of oxidative stress results in a number of beneficial effects (32–34). Experimental CRF-accelerated atherosclerosis was prevented by treatment with the antioxidant N-acetylcysteine, and plasma urea level was the only significant predictor of aortic plaque area fraction in a mouse model of CRF-accelerated atherosclerosis (4).

In cultured MCD3 cells, high urea concentration increased levels of ROS and the oxidative stress marker 8-oxoguanine (5). Based upon these findings, we hypothesized that elevated urea levels could be a contributing factor linking CRF, oxidative stress, insulin resistance, and atherosclerosis.

In the present study, we have shown that urea at concentrations seen in early and late CRF induce ROS production in cultured 3T3-L1 adipocytes, which increases modification of insulin signaling molecules by O-GlcNAc and reduces insulin-stimulated IRS and Akt phosphorylation and glucose transport. These negative signaling changes directly correlate with decreased insulin sensitivity and elevated levels of insulin resistance–associated adipokines. Similarly, uremic mice display increased ROS production, modification of insulin signaling molecules by O-GlcNAc, and increased expression of the insulin resistance–associated adipokines RBP4 and resistin in visceral adipose tissue. Moreover, uremic mice are insulin resistant and glucose intolerant. Treatment with the SOD/catalase mimetic (MnTBAP) again prevented urea-induced insulin resistance and glucose intolerance. Figure 8 Treatment of uremic mice with a SOD/catalase mimetic normalizes insulin resistance and glucose intolerance. Treated uremic mice and treated controls received MnTBAP i.p. for 2 weeks. (A) Insulin tolerance test. Mice were injected i.p. with insulin (0.75 U/kg), and blood glucose was measured at 0, 30, and 60 minutes. (B) i.p. glucose tolerance test. Mice were injected i.p. with glucose (1 mg/g body weight), and blood glucose was measured at 0, 15, 30, 60, and 120 minutes. (C) Insulin resistance in uremic mice. Insulin values from the IPGTT animals shown in B were determined by ELISA. (D) Glucose intolerance in uremic mice. Glucose values from B at 0 and 120 minutes. All results represent mean ± SEM of 7 animals per group. *P < 0.01 compared with controls.
and normalized insulin resistance–associated adipokine levels. Taken together, these data demonstrate that the proximate cause of insulin resistance in the urea-infused animal is ROS and that under these conditions, urea alone is sufficient to induce the same degree of insulin resistance as that seen in CRF.

The findings reported here may also provide a molecular explanation for the insulin resistance of CRF. Since urea can increase carbamylation and carbonylation of intracellular proteins (5), it is likely that these posttranslational modifications are responsible for altered function of cytosolic, nuclear, and mitochondrial proteins involved in the regulation of mitochondrial ROS production. Like urea, hyperglycemia also increases intracellular oxidative stress, modification of insulin signaling molecules by O-GlcNAc, and insulin resistance in arterial endothelial cells (35). Additive or synergistic effects of urea and hyperglycemia might explain the 2-fold increase in 5-year mortality of ESRD patients with diabetes compared with those without diabetes (36).

In summary, the observations reported here show that increased levels of urea, long considered to have negligible toxicity in patients with CRF, can cause insulin resistance in vitro and in vivo as a consequence of increased ROS generation. Treatment with a SOD/catalase mimetic normalizes insulin resistance and glucose intolerance in mice with CRF. Urea infusion in normal animals induces insulin resistance and elevates insulin resistance–associated adipokines, and treatment with a SOD/catalase mimetic prevents these urea-induced abnormalities. Because insulin resistance is an important independent cardiovascular risk factor, novel therapeutics that directly target urea-induced ROS and insulin resistance may potentially help reduce the high morbidity and mortality caused by ESRD.

### Methods

**Materials.** DMEM, nonessential amino acids, FBS, antibiotics, insulin, and urea were from Sigma-Aldrich. The urea used in these experiments was certified to be free of LPS and heavy metals. MnTBAP was from Calbiochem; protein A–sepharose and CM-H2DCFDA were from Invitrogen. Plasma insulin and resistin were measured with monoclonal antibody–based sandwich enzyme–linked immunosorbent assays (Linco Research). Antibodies and reagents used were as follows: anti-phosphotyrosine, anti–IRS-1, anti–pS636–IRS-1 (UBI); anti-Akt, anti–S473-Akt (Cell Signaling Technology); anti–O-linked glycosylation RL-2 (Affinity Bioreagents); and anti–3-nitrotyrosine (Upstate).

**Figure 9**

Treatment of uremic mice with a SOD/catalase mimetic normalizes oxidative stress, IRS-1 O-GlcNAc modification, and IRS-1 pTyr in epididymal fat. Treated uremic mice and treated controls received MnTBAP i.p. for 2 weeks. (A) Oxidative stress was quantified by immunoblotting of epididymal adipose tissue protein using anti–3-nitrotyrosine antibody and normalized for total protein by blotting with anti–β-actin antibody. (B) IP: Western blot analysis of uremia-induced O-GlcNAc modification of IRS-1 and (C) IRS-1 Tyr phosphorylation in epididymal adipose tissue. Levels of IRS-1 modification in epididymal adipose tissue from untreated control mice are shown as 100% in bar graphs. n = 5, *P < 0.01 compared with controls. Data represent mean ± SEM.

**Figure 10**

Treatment of uremic mice with a SOD/catalase mimetic normalizes insulin resistance–associated adipokine levels in adipose tissue and in plasma. Treated uremic mice and treated controls received MnTBAP i.p. for 2 weeks. (A) Relative expression of RBP4 and resistin in epididymal adipose tissue of untreated and MnTBAP-treated control mouse and in the plasma of untreated and MnTBAP-treated CRF mice by qPCR. The control values are shown as 1.0. (B) Plasma levels of RBP4 and resistin measured in the plasma of the untreated and MnTBAP-treated control mouse and in the plasma of untreated and MnTBAP-treated CRF mice by semiquantitative Western blot or ELISA, respectively. Each bar represents the mean ± SEM of 7 mice per group. *P < 0.01 compared with controls.
Mouse models. C57BL/6J wild-type mice (Harlan Italy S.r.l.) were housed in a temperature-controlled facility (21°C–23°C) with a 12-hour light/12-hour dark cycle. The mice were allowed free access to food and water. The experiments were performed according to the principles stated in the Italian law on animal experiments and were approved by the Animal Experiments Inspectorate, Ministry of University, Rome, Italy.

At 7 weeks of age, the mice were randomly allocated to 5/6 nephrectomy and no surgery. A mixture of 0.079 mg/ml fentanyl, 2.5 mg/ml fluanisone, and 1.25 mg/ml midazolam (Hypnorm/Dormicum) at a dose of 0.08 to 0.10 ml/10 g body weight was given subcutaneously for anesthesia. Moderate uremia was induced by a 2-step surgical procedure (37). In brief, the upper and lower poles of the right kidney were resected. One week later, the left kidney was removed after ligation of the renal blood vessels and the ureter. Control animals were subjected to a sham operation that included decapsulation of both kidneys. Special care was taken to avoid damage to the adrenals. A randomized group of 5/6 nephrectomized mice and their controls were injected i.p. with MnTBAP at a dose of 10 mg/kg for 15 days starting immediately after the second surgical procedure. For measuring food intake, mice were housed individually and food intake was estimated by measuring the weight of powdered food remaining in feeding chambers designed to maximize spill capture. After the first surgery, the CFR mice were fed ad libitum with powdered mouse diet (LabDiet) that was weighed and replaced daily, and control mice were pair-fed with CFR mice. Body weight was monitored every week. Plasma fasting insulin levels were determined weekly using monoclonal antibody–based sandwich enzyme–linked immunosorbent assays (Linco Research). Three weeks after the first surgical procedure, animals underwent glucose and insulin tolerance tests. The following day, after terminal blood sampling, animals were killed with a lethal dose of sodium pentobarbital (100 mg/kg; Nembutal, Abbott Laboratories).

Blood chemistry. Mouse blood glucose was determined by testing 5 μl of tail-vein blood using an Accu-Chek (Roche) active blood glucose–monitoring system. Every week, BUN was analyzed using the L-Type UN Kit (Wako) based on an enzymatic assay utilizing urease and glutamate dehydrogenase. Plasma insulin and plasma resistin were measured with monoclonal antibody–based sandwich enzyme–linked immunosorbent assays (Linco Research or R&D Systems Europa). Plasma RBP4 was determined by semiquantitative Western blot using anti-RBP4 antibody (Alexis Biochemical).

Insulin and glucose tolerance tests. Insulin and glucose tolerance tests were performed as described previously (38). Blood glucose was determined by Accu-Chek (Roche) active blood glucose–monitoring system, and plasma insulin levels were measured by ELISA (Linco Research).

Cell culture. 3T3-L1 murine fibroblasts were propagated and differentiated as described previously (39). In brief, the cells were propagated in FBS (DMEM containing 5 mM glucose, 10% FBS, and penicillin/streptomycin [100 units/ml each]) and allowed to reach confluence. After 2 days (day 0), the medium was changed to DM1 (containing FBS and 160 nM insulin, 250 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine). Two days later (day 2), the medium was switched to DM2 (FBS containing 160 nM insulin). After another 2 days, the cells were switched back to FBS for 2 days, and then they were incubated in medium containing 20 mM urea for 48 hours. Mannitol was used as an osmotic control for the highest urea concentration. To test urea toxicity effect on differentiated 3T3-L1, cell toxicity was determined using a Cell Toxicity Colorimetric Assay Kit from Sigma-Aldrich.
Measurement of [1-3H]2-deoxyglucose uptake. [3H]2-deoxyglucose uptake was performed as described previously (39). In brief, the cells were glucose starved for 30 minutes. Insulin was then added for 5 minutes, followed by addition of the labeled glucose in the continued presence of the indicated amount of insulin. Glucose transport assays were carried out in a volume of 1 ml (Kreb’s-Ringer/HEPES buffer, pH 7.4) in a 3.5-cm dish for 25 minutes at 37°C. The concentration of 2-deoxyglucose was 50 μM with 0.33 μCi of [3H]2-deoxyglucose/ml. After terminating the transport assay with 3 washes of ice-cold assay buffer, each plate was solubilized in 1 ml of 0.1 M NaOH and quenched with 50 μl of concentrated HCl. A 0.9-ml aliquot was removed for determination of radioactivity by liquid scintillation counting, and 50 μl aliquots were used for measurement of protein using a bicinchoninic acid (BCA) assay. Glucose uptake remained linear within the first 30 minutes. Each measurement at the indicated urea concentrations represents the average of 8 independent measurements performed on 5 separate occasions with different cell preparations.

Immunoprecipitation and Western blotting. At the end of treatments, 3T3-L1 adipocytes were incubated in the presence or absence of 5 nM insulin for 10 minutes. Epididymal adipose tissue was isolated from control and anti-β2-ADR antibodies with a 50% slurry of protein A agarose beads overnight at 4°C. After washing with buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, and 0.5% deoxycholate with protease inhibitors, proteins were released and separated on 10% SDS-PAGE gels. Membranes blotted with the antibodies were then probed with far-advanced renal failure. Mayo Clin Proc. 1987;24(7):1-21.  