Impaired Vasodilator Activity in Deoxycorticosterone Acetate-Salt Hypertension Is Associated With Increased Protein O-GlcNAcylation

Victor V. Lima, Fernanda R.C. Giachini, Hyehun Choi, Fernando S. Carneiro, Zidonia N. Carneiro, Zuleica B. Fortes, Maria Helena C. Carvalho, R. Clinton Webb, Rita C. Tostes

Abstract—Hyperglycemia, which increases O-linked β-N-acetylglucosamine (O-GlcNAc) proteins, leads to changes in vascular reactivity. Because vascular dysfunction is a key feature of arterial hypertension, we hypothesized that vessels from deoxycorticosterone acetate and salt (DOCA-salt) rats exhibit increased O-GlcNAc proteins, which is associated with increased reactivity to constrictor stimuli. Aortas from DOCA rats exhibited increased contraction to phenylephrine (E_max [mN]=17.6±4 versus 10.7±2 control; n=6) and decreased relaxation to acetylcholine (47.6±6% versus 73.2±10% control; n=8) versus arteries from uninephrectomized rats. O-GlcNAc protein content was increased in aortas from DOCA rats (arbitrary units=3.8±0.3 versus 2.3±0.3 control; n=5). PugNAc (O-GlcNAcase inhibitor; 100 μmol/L; 24 hours) increased vascular O-GlcNAc proteins, augmented phenylephrine vascular reactivity (18.2±2 versus 10.7±3 vehicle; n=6), and decreased acetylcholine dilation in uninephrectomized (41.4±26 versus 73.2±3 vehicle; n=6) but not in DOCA rats (phenylephrine, 16.5±3 versus 18.6±3 vehicle; n=6; acetylcholine, 44.7±8 versus 47.6±7 vehicle, n=6). PugNAc did not change total vascular endothelial nitric oxide synthase levels, but reduced endothelial nitric oxide synthase Ser-1177 and Akt Ser-473 phosphorylation (P<0.05). Aortas from DOCA rats also exhibited decreased levels of endothelial nitric oxide synthase Ser-1177 and Akt Ser-473 (P<0.05) but no changes in total endothelial nitric oxide synthase or Akt. Vascular O-GlcNAc–modified endothelial nitric oxide synthase was increased in DOCA rats. Blood glucose was similar in DOCA and uninephrectomized rats. Expression of O-GlcNAc transferase, glutamine:fructose-6-phosphate amidotransferase, and O-GlcNAcase, enzymes that directly modulate O-GlcNAcylation, was decreased in arteries from DOCA rats (P<0.05). This is the first study showing that O-GlcNAcylation modulates vascular reactivity in normoglycemic conditions and that vascular O-GlcNAc proteins are increased in DOCA-salt hypertension. Modulation of increased vascular O-GlcNAcylated protein may represent a novel therapeutic approach in mineralocorticoid hypertension. (Hypertension. 2009;53:166-174.)

Key Words: O-linked N-acetylglucosaminylation ■ DOCA-salt ■ eNOS ■ vascular reactivity

Arterial hypertension is a multifactorial condition estimated to affect 25% of the adult population or ~1 billion people worldwide. The prevalence of hypertension is predicted to increase to 30% by 2025 (ie, ~1.56 billion people will harbor this condition in the next several years). Considered a major risk factor for cardiovascular disease, which is the leading cause of morbidity and mortality in most Western countries, arterial hypertension is also responsible for >7 million deaths. In addition, estimates of expenditures related to hypertension and its complications in 2007 indicate that total direct costs will approach $66.4 billion. Prevention of hypertension and hypertension-associated end-organ damage is one of the major therapeutic aims in cardiovascular medicine. Although current drugs are effective in many patients, there are still a great number of patients that are unresponsive even to combination therapy. This clearly indicates that new advances in the field are required.

Abnormal vascular reactivity, including impaired endothelium-dependent relaxation and enhanced sensitivity to vasoconstrictors, is a hallmark of hypertensive disease. However, our current understanding of the cellular and molecular mechanisms leading to vascular dysfunction in hypertension is still incomplete. Many proteins important in cardiovascular function, including kinases, phosphatases, transcription factors, and cytoskeleton proteins, have been identified as targets for O-linked β-N-acetylglucosamine (O-GlcNAcylation), a post-translational modification that influences protein expression, degradation, and trafficking.

Although it is clear that O-GlcNAcylation plays a critical role in the regulation of cell function, there is a
paucity of information on the vascular effects of O-GlcNAcylation. Preliminary evidence from our laboratory suggests that increased O-GlcNAc levels are associated with increased reactivity to constrictor stimuli and impaired endothelium-dependent vasodilatation, which are all important in hypertension-associated vascular dysfunction. However, no studies have addressed whether chronically elevated vascular levels of O-GlcNAc proteins represent a common mechanism underlying the adverse effects of hypertension on vascular function. We hypothesized that augmented O-GlcNAcylation in arteries from deoxycorticosterone acetate and salt (DOCA-salt) hypertensive animals reduces vasodilator activity and increases reactivity to constrictor stimuli, contributing to abnormal vascular reactivity in mineralocorticoid hypertension.

To address our hypothesis, we first determined the vascular content of O-GlcNAc proteins in hypertensive rats. We then evaluated the effects of increased O-GlcNAc levels on vascular reactivity in arteries from control and DOCA hypertensive animals. A pharmacological approach (incubation of vessels with a potent inhibitor of O-GlcNAcase6,7) was used to increase O-GlcNAc protein levels.

Figure 1. O-GlcNAc proteins content is increased in arteries from DOCA-salt rats and after treatment with PugNAc. A, Aorta. Representative Western blot image of O-GlcNAc proteins (top) and corresponding bar graphs showing the relative content of O-GlcNAc proteins after normalization to β-actin expression (bottom). B, Mesenteric arteries. Representative Western blot image of O-GlcNAc proteins (top) and corresponding bar graphs showing the relative content of O-GlcNAc proteins after normalization to β-actin expression (bottom). Results are presented as mean±SEM for n=6 in each experimental group. *P<0.05 vs vehicle; †P<0.05 vs Uni.

Figure 2. PE-induced contractile response is increased in aortas from DOCA-salt rats and after treatment with PugNAc. A, Incubation with PugNAc increases vascular contraction to PE in rat thoracic aorta from Uni rats (n=6) vs vehicle (methanol)-treated aortas (n=6). B, PugNAc incubation does not change PE-induced contraction in arteries from DOCA rats (n=6). Experimental values of contraction were calculated relative to the contractile response produced by 120 mmol/L KCl, which was taken as 100%. Results are presented as mean±SEM in each experimental group. *P<0.05 vs Uni; †P<0.05 vs vehicle.
Methods

Animals
Male Wistar rats weighing 230 to 250 g at 8 weeks of age (Harlan Laboratories; Indianapolis, Ind) were used in this study. All procedures were performed in accordance with the guiding principles in the care and use of animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed 4 per cage on a 12-hour light/dark cycle and fed a standard chow diet with water ad libitum. Details of the methods can be found in an online supplement available at http://www.hypertensionaha.org.

DOCA-Salt Hypertension and Systolic Blood Pressure Measurements
DOCA-salt hypertension and systolic blood pressure (SBP) measurements were performed as described previously.9 At the end of 5 weeks of treatment, vessels were removed and submitted to experimental procedures. To determine a possible relationship between increments in blood pressure and O-GlcNAc modification of vascular proteins, DOCA-salt treatment was performed, and vessels collected for Western blot analysis also at 1, 2, and 3 weeks.

Vascular Functional Studies
After euthanasia, thoracic aorta and second-order mesenteric arteries were removed and cleaned from fat tissue in an ice-cold physiological salt solution. Arterial segments were incubated in Eagle’s minimum essential medium containing l-glutamine (1%), fetal bovine serum (10%), and penicillin and streptomycin (0.5%), and incubated with vehicle (methanol) or PugNAc [O-(2-acetamido-2-deoxy-o-glucopyranosylidene) amino-N-phenylcarbamate; 100 μmol/L] for 24 hours. After incubation, vessels (4 mm in length) were carefully mounted as ring preparations in standard organ chambers for isometric tension recordings, as described previously.9

Concentration–response curves to phenylephrine (PE; 1 mmol/L to 100 mmol/L) were performed in the presence and absence of Nω-nitro-l-arginine methyl ester (l-NAME; 100 μmol/L). Endothelium-dependent and endothelium-independent relaxation was assessed by measuring the relaxation response to acetylcholine (Ach; 1 nmol/L to 100 μmol/L) and sodium nitroprusside (0.1 mmol/L to 10 mmol/L), respectively, in PE-contracted vessels (1 μmol/L).

Western Blot Analysis
Proteins (60 μg) extracted from aorta or mesenteric arteries were separated by electrophoresis and Western blots performed as described previously.9 Antibodies were as follows: anti–O-GlcNAc antibody, CTD 110.6 (1:2000; Pierce Biotechnology), total endothelial nitric oxide synthase (eNOS), Akt, and phosphatidylinositol 3-kinase (PI3K; 1:100; Cell Signaling Technology, Inc). Immunoblots for nonphosphoproteins were performed in the same membranes used to evaluate their phosphorylated forms: p-eNOS (Ser 1177), p-Akt (Ser 473), and p-PI3K (Tyr 458; 1:400; Cell Signaling Technology, Inc). Antibody CTD 110.6 was used to detect O-GlcNAc proteins in arteries (thoracic aorta and second-order resistance mesenteric) from DOCA-salt and Uni rats.

Immunoprecipitation Analysis
Immunoprecipitation was performed as described previously10 with anti-eNOS (1:1000; Cell Signaling Technology, Inc).

Data Analysis
Results are shown as mean±SEM, and n represents the number of animals used in the experiments. Contractions were recorded as changes in the displacement (mN) from baseline. Relaxation is expressed as percentage change from the PE contracted levels. Concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software). Statistical analyses of Emax and pD2 values were performed using a nonlinear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software). Values of P<0.05 were considered statistically significant.

Results
At 5 weeks of treatment, SBP (mm Hg) was higher in DOCA-salt rats (196±4) compared with their respective controls (110±4). DOCA-salt rats also exhibited decreased body weight compared with normotensive rats (319±7 g versus 390±14 g, respectively). Plasma glucose (mg/dL) was similar between uninephrectomized (Uni) and DOCA-salt rats (100.3±3.2 versus 98.9±7.4, respectively).

To test the hypothesis that increased O-GlcNAcylation contributes to increased vascular reactivity in mineralocorticoid hypertensive animals, we first determined the content of O-GlcNAc proteins in arteries (thoracic aorta and second-order resistance mesenteric) from DOCA-salt and Uni rats.

The vascular content of O-GlcNAc proteins was increased in DOCA-salt hypertensive animals, we first determined the content of O-GlcNAc proteins in arteries (thoracic aorta and second-order resistance mesenteric) from DOCA-salt and Uni rats.

Vascular reactivity was measured in the presence of O-GlcNAc-modified proteins and compared with their respective controls. Statistical analyses of Emax and pD2 values were performed using 1-way ANOVA or Student t test. Post hoc comparisons were performed using Newman–Keuls test. Western blot data were analyzed by 1-sample t test, and the P value was computed from the t ratio and the degrees of freedom. Values of P<0.05 were considered statistically significant.

Publications

Table. Emax and pD2 Values for PE, Ach, and Sodium Nitroprusside–Induced Responses in Aortas From Control and DOCA-Salt Rats

<table>
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<tr>
<th>Agonist</th>
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<td></td>
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<td>DOCA-Salt</td>
<td>Uni</td>
<td>DOCA-Salt</td>
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<td>PE</td>
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<td>PE+l-NAME</td>
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<tr>
<td>PE+PugNAc</td>
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<td>7.4±0.01†</td>
<td>123±22</td>
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<td>PE+PugNAc+l-NAME</td>
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<tr>
<td>ACh</td>
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<tr>
<td>ACh+PugNAc</td>
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Values are means±SEM for n experiments in each group. *P<0.05 vs Uni; †P<0.05 vs vehicle-treated.
from Uni or DOCA-salt rats, after incubation with 10^{-4} mol/L L-NAME (Table).

Similar results were obtained with small mesenteric arteries. As shown in Figure 3A and 3B, small mesenteric arteries from DOCA-salt rats displayed increased contraction to PE compared with Uni. After 24-hour incubation with PugNac, the O-GlcNAcase inhibitor increased PE-induced contraction in arteries from Uni (Figure 3A) but not in those from DOCA-salt rats (Figure 3B). Furthermore, L-NAME incubation abolished differences among the groups (Figure 3C) similarly to what was observed in aortas from Uni and DOCA-salt rats (Table).

Aortas from DOCA-salt rats displayed decreased relaxation to ACh when compared with arteries from Uni rats (Figure 4A). In addition, PugNac incubation impaired ACh-induced relaxation in arteries from control (Figure 4A) but not DOCA-salt rats (Figure 4B). No differences in vascular reactivity to sodium nitroprusside were observed between arteries from Uni and DOCA-salt hypertensive rats or between arteries treated with vehicle or PugNac (Table).

Considering that increased O-GlcNAcylation leads to decreased levels of phosphorylated eNOS^{Ser-1177}, we determined whether changes in vascular reactivity after treatment with PugNac and DOCA-salt hypertension are related to changes in the expression and activity (indicated by phosphorylation levels) of enzymes in the eNOS/Akt/PI3K pathway.
Our data show that arteries from DOCA-salt rats display decreased phosphorylation of eNOS at Ser1177 (Figure 5A) as well as Akt at Ser473 (Figure 5B) but not PI3K at Tyr458 (Figure 5C). Total expression of eNOS, Akt, or PI3K was not modified in DOCA-salt rats (Figure 5A, 5B, and 5C, respectively). Similarly, increased O-GlcNAcylation (treatment with PugNac) leads to decreased phosphorylation of eNOS and Akt but not PI3K in arteries from control animals (Figure 6A, 6B, and 6C, respectively).

Immunoprecipitation analysis was performed to evaluate whether eNOS is indeed a target for O-GlcNAc modification. O-GlcNAc proteins were immunoprecipitated and labeled with anti-eNOS. As shown in Figure 7, aortas from DOCA-salt rats exhibit increased expression levels of O-GlcNAc–modified e-NOS compared with aortas from Uni. This result shows that during mineralocorticoid hypertension, eNOS protein is an important target for O-GlcNAc modification.

To determine possible mechanisms associated with increased vascular levels of O-GlcNAc proteins in DOCA-salt hypertension, we evaluated the expression of OGT and O-GlcNAcase, enzymes that directly regulate O-GlcNAcylation, as well as the enzymes that directly regulate O-GlcNAcylation.
expression of GFAT, the rate-limiting enzyme in the hexosamine biosynthetic pathway.

As shown in Figure 8, OGT, O-GlcNAcase, and GFAT expression are decreased in aortas from DOCA-salt rats. Treatment with PugNAc induced a similar reduction in OGT levels but did not modify O-GlcNAcase or GFAT expression in arteries from Uni rats.

To clarify whether there is a relationship between increased blood pressure levels, augmented vascular O-GlcNAc protein content, and changes in expression levels of the enzymes OGT and O-GlcNAcase, these parameters were determined at different intervals (1, 2, 3, or 5 weeks) of mineralocorticoid hypertension. As shown in Figure 9A, SBP progressively increased from days 14 to 35 of DOCA-salt treatment. SBP values were maintained at the same levels at 5 weeks of treatment. A similar profile was observed for the vascular levels of O-GlcNAc–modified proteins (ie, aortas from DOCA-salt rats) or O-GlcNAc proteins after 14 days; these levels were further augmented after 21 days of treatment; Figure 9B) and maintained after 5 weeks. An inverse temporal correlation was observed for O-GlcNAcase and OGT expression (Figure 9C and 9D, respectively). The expression of both enzymes was decreased after 14 days of DOCA-salt treatment. After 21 days, a further reduction was observed and maintained until 5 weeks of treatment. No changes in SBP, vascular O-GlcNAc levels, O-GlcNAcase, or OGT expression were observed in Uni rats.

**Discussion**

O-GlcNAc on serine and threonine residues of nuclear and cytoplasmic proteins is a highly dynamic post-translational modification that plays a key role in signal transduction pathways. Numerous proteins, including kinases, phosphatases, transcription factors, and cytoskeleton proteins, have been identified as targets of O-GlcNAc modification. Although it is clear that O-GlcNAcylation plays a critical role in the regulation of cell function, there is a paucity of information on the vascular effects of O-GlcNAcylation. Preliminary evidence suggests that increased O-GlcNAc levels may be associated with increased cell proliferation, impairment of endothelial and vascular smooth muscle cell function, as well as increased fibrosis and inflammation, which are all important in hypertension-associated vascular dysfunction.

Our data show for the first time that the vascular content of O-GlcNAc proteins is increased in arteries (thoracic aorta and second-order resistance mesenteric) from DOCA-salt hypertensive animals. In addition, PugNAc incubation, which increased vascular content of O-GlcNAc proteins in arteries from control animals, had a minor effect on arteries from DOCA-salt rats. Furthermore, whereas PugNAc increased vascular reactivity to the α-1 agonist PE and decreased ACh vasodilation in control Uni animals, it did not change vascular reactivity to PE or ACh, which was already impaired in arteries from DOCA-salt rats. Based on these observations, one may suggest that chronically elevated levels of O-GlcNAc represent a common mechanism underlying the adverse effects of hypertension on vascular function, thereby contributing to hypertension-associated vascular dysfunction.

Considering that PugNAc effects on PE reactivity were not observed in the presence of l-NAME (inhibitor of NOS activity) and that PugNAc, as well as mineralocorticoid hypertension, did not change sodium nitroprusside response, we determined whether increased levels of O-GlcNAcylation modify the PI3K/Akt/eNOS pathway.

We observed that total eNOS expression was unchanged in arteries from DOCA rats, whereas phosphorylated eNOS levels were decreased. In a study by Callera et al, it was reported that eNOS expression was elevated in DOCA-salt hypertension, but this study also reported similar observations on reduced phosphorylated eNOS. Furthermore, as demonstrated by Northcott et al, we found similar concentrations of total Akt in arteries from Uni and DOCA-salt rats but significantly lower phosphor-Akt protein density in DOCA-salt arteries. It is possible that increased O-GlcNAc levels mediate the decreased vascular Akt/eNOS activity in DOCA-salt arteries. It is possible that increased O-GlcNAc levels mediate the decreased vascular Akt/eNOS activity in mineralocorticoid hypertension. Our data showing that PugNAc decreased levels of phosphorylated eNOS and Akt further support this hypothesis. Although increased PI3K activity has been suggested to play a role in spontaneous tone development and hyper-reactivity in DOCA-salt aortas, no differences in the total or phosphorylated forms of PI3K were observed between arteries from Uni and DOCA-salt rats. In addition, PugNAc treatment did not change total or phosphorylated PI3K. Altogether, these results support the hypothesis that augmented O-GlcNAcylation increases vascular reactivity to contractile stimuli via decreased eNOS activity and, therefore, contributes to abnormal vascular reactivity in mineralocorticoid hypertension.

Other reports have shown that O-GlcNAcylation interferes with the eNOS pathway. Under high glucose conditions and increased O-GlcNAcylation, phosphorylation of eNOS at Ser\textsuperscript{1177} (eNOS\textsuperscript{Ser1177}) is decreased both in vascular and penile tissue from diabetic animals. Furthermore, Du et al showed a possible mechanism underlying impaired endothelium-dependent vasodilation in patients with diabetes, a pathology where O-GlcNAc levels are increased. They showed that in cultured cells, hyperglycemia is able to inhibit eNOS activity and increase O-GlcNAc modification of the eNOS protein after O-GlcNAc immunoprecipitation. Results are presented as mean±SEM in each experimental group (n=4 each group). *P<0.05 vs Uni.

Figure 7. Expression of O-GlcNAc–modified eNOS protein is augmented in aortas from DOCA-salt rats. O-GlcNAc proteins were immunoprecipitated and Western blot was performed on eNOS proteins. Top, Representative images of Western blot. Bottom, Bar graphs showing the relative expression of eNOS protein after O-GlcNAc immunoprecipitation. Results are presented as mean±SEM in each experimental group (n=4 each group).
enzyme with a reciprocal decrease in phosphorylation. This was blocked by inhibition of GFAT, suggesting that flux through the hexosamine biosynthetic pathway was a contributing factor. However, increased vascular O-GlcNAcylation in mineralocorticoid hypertension does not seem positively associated with vascular increased glucose flux through the hexosamine biosynthetic pathway because blood glucose levels were similar in DOCA and control rats. In addition, decreased expression of GFAT was observed in vessels from DOCA-salt hypertensive rats.

O-GlcNAc is considered similar to protein phosphorylation (O-phosphate attachment) in that both modifications occur on serine and threonine residues, both are dynamically added and removed from proteins in response to cellular signals, and

Figure 8. Vascular expression of OGT (A), O-GlcNAcase (B), and GFAT (C) is decreased in DOCA-salt hypertension. Top, Representative Western blot image of OGT, O-GlcNAcase, and GFAT. Bottom, Corresponding bar graphs showing the relative expression of OGT, O-GlcNAcase, and GFAT after normalization to β-actin expression. Results are presented as mean±SEM for n=4 in each experimental group. *P<0.05 vs Uni or vehicle (methanol).
both alter the function and association of the modified protein. Many phosphorylation sites are also known glycosylation sites, and this reciprocal occupancy may produce different activities or alter stability in the protein. Therefore, one potential mechanism by which O-GlcNAcylation may change vascular reactivity includes the complex interplay between O-GlcNAcylation and phosphorylation. In support of this hypothesis, decreased phosphorylation of eNOS at Ser1177 (eNOS\textsuperscript{Ser1177}) induced by O-GlcNAcylation of the enzyme, has been reported in vessels from diabetic animals\textsuperscript{11,13} and after platelet aggregation induced by advanced glycation end-products.\textsuperscript{18}

To further determine mechanisms associated with increased vascular O-GlcNAcylation, OGT and O-GlcNAcase expression were determined. The cycling of O-GlcNAc on serine or threonine residues of target proteins is controlled by 2 highly conserved enzymes: OGT (or uridine diphospho-N-acetyl glucosamine: polypeptide β-N-acetylglucosaminyl transferase; UDP-Nac transferase) and β-N-acetylglucosaminidase (O-GlcNAcase). Whereas OGT catalyzes the addition of O-GlcNAc to the hydroxyl group of serine or threonine residues of a target protein, O-GlcNAcase catalyzes the hydrolytic cleavage of O-GlcNAc from post-translationally modified proteins.\textsuperscript{4} The overall catalytic activity of OGT is controlled by the concentration of its donor substrate UDP–GlcNAc is highly sensitive to flux in nutrients and energy, mainly through the hexosamine biosynthetic pathway. Increased flux through the pathway, either through increased glucose uptake or glucosamine treatment, which is distal to GFAT, increases the production of UDP–GlcNAc, and stimulates O-GlcNAc modification of proteins. GFAT is the rate-limiting enzyme of the pathway, converting fructose 6-phosphate to glucosamine 6-phosphate, with glutamine as the amine donor. Expression of O-GlcNAcase, OGT, and GFAT was decreased in DOCA aortas. Whereas increased vascular O-GlcNAcylation in mineralocorticoid hypertension may be mediated by decreased O-GlcNAcase expression/activity, the concomitant decrease in OGT and GFAT may represent a compensatory mechanism for the augmented vascular levels of O-GlcNAc proteins. In other words, whereas decreased O-GlcNAcase expression/activity may be a causal effect of augmented O-GlcNAc proteins, decreased OGT and GFAT expression/activity may represent counter-regulatory mechanisms. Interestingly, a temporal relationship was observed not only between increased blood pressure and the higher vascular O-GlcNAcylation content, but also between augmented O-GlcNAc levels and decreased expression of O-GlcNAcase and OGT. We are currently investigating whether mineralocorticoid hormones themselves or other factors associated with DOCA-salt hypertension mediate the changes in O-GlcNAc expression/OGlcNAcylation.

In summary, the present study shows that O-GlcNAcylation modulates vascular reactivity in normoglycemic conditions and that vascular O-GlcNAc proteins are increased in DOCA-salt hypertension. Modulation of increased vascular O-GlcNAcylation, which is associated with decreased eNOS activity and augmented reactivity to constrictors stimuli, may represent a novel therapeutic approach in mineralocorticoid hypertension.

Figure 9. A time course study showing a temporal relationship among increased BP, vascular O-GlcNAcylation, and decreased expression of O-GlcNAcase and OGT. A, SBP. B, Vascular O-GlcNAc proteins. C, O-GlcNAcase. D, OGT levels in Uni (gray bars; n=6) and DOCA-salt rats (black bars; n=6), measured at different time points: after 7, 14, 21, and 35 days of DOCA-salt treatment. Results are presented as mean±SEM for each experimental group. *P<0.05 vs Uni.
Perspectives
The incidence of hypertension in the US population is very high, and a variety of pharmacological preparations are available for therapy. Unfortunately, despite these options, vascular dysfunction persists in many patients, and end-organ injury remains a serious complication. Recent studies demonstrated that many proteins important in cardiovascular function are targets for O-GlcNAcylation. Because our preliminary data strongly support our working hypothesis, we are speculating that O-GlcNAcylation signaling pathway may be a novel therapeutic target for the treatment of hypertension. For the first time, we have shown that augmented O-GlcNAc levels directly affect proteins related to vascular relaxation during mineralocorticoid hypertension. Our next step will be to investigate whether contractile proteins, such as proteins from RhoA/Rho kinase pathway, are regulated by O-GlcNAc modifications and whether these changes contribute to increased vascular responses to constrictor stimuli.

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Disclosures
None.

References
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Impaired vasodilator activity in DOCA-salt hypertension is associated with increased protein O-GlcNAcylation

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Short title: O-GlcNAc and vascular reactivity in hypertension

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vlima@mcg.edu
MATERIAL FOR THE "ONLINE SUPPLEMENT":
Expanded materials and methods.

Animals:

Male Wistar rats (8 weeks-old, 230–250g; Harlan Laboratories, Indianapolis, IN) were used in this study. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed four per cage on a 12-h light/dark cycle and fed a standard chow diet with water ad libitum.

DOCA-salt hypertension and systolic blood pressure measurements:

All animals were uninephrectomyzed under anesthesia with a mixture of ketamine (80mg·kg⁻¹) and xylazine (10mg·kg⁻¹). Uninephrectomyzed animals were given no further treatment (Uni-rats) or received 1% NaCl plus 0.2% KCl in the drinking water and DOCA-salt silastic pellets (0.2g·kg⁻¹ for rats), which were implanted subcutaneously at the scapular region (DOCA-rats). The duration of treatment was 5 weeks.

Systolic blood pressure (SBP) was measured weekly in conscious animals by tail cuff method using a RTBP1001 blood pressure system (Kent Scientific Corporation Connecticut). At the end of 5 weeks of treatment, vessels were removed and they were submitted to experimental procedures. To determine a possible relationship between increments in blood pressure and O-GlcNAc modification of vascular proteins, DOCA-salt treatment was interrupted and vessels collected for Western blot analysis also at 1, 2 and 3 weeks.

Vascular functional studies:

After euthanasia, the thoracic aorta and second-order mesenteric arteries were removed and cleaned from fat tissue in an ice-cold physiological salt solution (PSS), containing (mM): NaCl, 130; NaHCO₃, 14.9; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄·7H₂O 1.18; CaCl₂·2H₂O, 1.56, EDTA, 0.026, glucose 5.5. Arterial segments were incubated in Eagle’s Minimum Essential Medium (EMEM) containing L-glutamine (1%), fetal bovine serum (10%), penicillin and streptomycin (0.5%), and incubated with vehicle (methanol) or PugNAc [O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate; 100µM] for 24h. Following incubation, vessels (4 mm in length) were carefully mounted as ring preparations in standard organ chambers for isometric tension recordings by a PowerLab 8/SP data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia). The tissue was continuously bubbled with 95% O₂ and 5% CO₂ and maintained at 37°C, under a resting tension (aortas, 30mN for rats). After a 45 min equilibration period arterial integrity was assessed first by
stimulation of vessels with potassium chloride (KCl - 120 mM) and, after washing and a new stabilization period, by contracting the segments with phenylephrine (PE; 1 M) followed by stimulation with acetylcholine (ACH; 10µM).

Concentration-response curves to PE (1nM to 100µM) were performed to evaluate vascular contractility, both in the presence and absence of a NO synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME 100 µM) for 40 minutes. Endothelium-dependent relaxation was assessed by measuring the relaxation response to ACh (1nM to 100 µM) and endothelium-independent relaxation was assessed by measuring the relaxation response to sodium nitroprusside (SNP 0.1nM to 10 µM) in PE-contracted vessels (1 µM).

**Western blot analysis:**

Proteins (60 µg) extracted from aorta or mesenteric arteries were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (10%) for 1 hour at 24°C. Membranes were then incubated with antibodies overnight at 4°C. Antibodies were as follows: anti-O-GlcNAc antibody, CTD 110.6 (1:2000; Pierce Biotechnology, USA), total eNOS, Akt and PI3K [(1:1000) Cell Signaling Technology, Inc]. Immunoblots for nonphosphoproteins were carried out in the same membranes used to evaluate their phosphorylated forms: p-eNOS (Ser 1177), p-Akt (Ser 473) and p-PI3K (Tyr 458) [(1:400); Cell Signaling Technology, Inc]. O-GlcNAc transferase (OGT) [1:400, Santa Cruz antibodies], glutamine:fructose-6-phosphate amidotransferase (GFAT) [1:350, Santa Cruz antibodies] and O-GlcNAc case protein expression was also evaluated. O-GlcNAcase antibody was kindly provided by Dr. Sidney Whiteheart (1:1000, University of Kentucky).

After incubation with secondary antibodies, signals were developed for chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results were normalized to beta-actin [(1:10000), Sigma-Aldrich, Inc] protein and expressed as arbitrary units.

**Immunoprecipitation analysis:**

Aortic segments were homogenized in lysis buffer (20 mM Tris–HCl, pH 7.4, 5 mM Na2P2O7, 100 mM NaF, 2 mM Na3 VO4, 1% NP-40, 1 lg/ml aprotinin, 1 lg/ml leupeptin, and 1 mM PMSF). Cell extracts (1 mg) were sonicated and cell debris was removed by centrifugation. Cell lysates were incubated with 2 µg of O-GlcNAc antibody, 40 µl of protein A/G plus agarose (Santa Cruz Biotechnology) overnight, at 4 °C, with rotation. The beads were collected and extensively washed with phosphate buffered saline (PBS). SDS–PAGE sampling buffer was added to the beads and the samples were boiled. Immunoblot was performed with anti-eNOS [(1:1000); Cell Signaling Technology, Inc]. After incubation with secondary antibodies, signals were developed for chemiluminescence, visualized by autoradiography, and quantified densitometrically.