Vasopressin Induces Vascular Superoxide Via Endothelin-1 in Mineralocorticoid Hypertension

Lixin Li, James J. Galligan, Gregory D. Fink, Alex F. Chen

Abstract—We have recently reported that endothelin-1 (ET-1), which is increased in the arteries of deoxycorticosterone acetate (DOCA)–salt hypertensive rats, stimulates superoxide production. However, the humoral mechanisms responsible for ET-1–induced superoxide formation in low-renin models of hypertension, such as DOCA-salt hypertension, remain undefined. Vasopressin is known to upregulate vascular preproET-1 gene expression in DOCA-salt rats, an effect that is absent in vasopressin-deficient Brattleboro rats treated with DOCA-salt. The present study tested the hypothesis that vasopressin contributes to ET-1–induced vascular superoxide production in DOCA-salt hypertensive rats. Carotid arterial segments of DOCA, sham (uninephrectomized), or normal (untreated) rats were used for the study. In vitro vasopressin treatment of carotid arteries from normal rats for 24 hours, but not 4 hours, increased both ET-1 and superoxide levels. The increase of vasopressin-induced superoxide was reduced by pretreatment of the vessels with ABT627, a selective ET, receptor antagonist ABT627. Vasopressin, ET-1, and superoxide levels were significant elevated in carotid arteries of DOCA-salt rats compared with sham controls. The selective V1-vasopressin receptor antagonist (β-Mercapto-β, β-cyclopentamethylenepropiony1, O-Me-Tyr2, Arg8 vasopressin, ME-AVP), decreased superoxide both in vasopressin-treated vessels of normal rats and in vessels of DOCA-salt rats, with a concomitant reduction of ET-1 content. These results suggest that vasopressin increases vascular superoxide levels by stimulating ET-1 formation in mineralocorticoid hypertension, and that V1-vasopressin receptors play an important role in this process. (Hypertension. 2003;41[part 2]:663-668.)

Key Words: vasopressin ■ endothelin ■ superoxide ■ hypertension, low renin

Substantial evidence suggests that increased levels of superoxide anion (O2−) in the blood vessel wall contribute to vascular dysfunction in a number of pathological processes, including atherosclerosis, diabetes, and hypertension. 1,3 O2− is produced in activated endothelial cells, smooth muscle cells, and adventitial fibroblasts. 6 Vascular dysfunction is manifested as impaired endothelium-dependent NO-mediated relaxation, 1,3 adhesion molecule expression, 7 low-density lipoprotein oxidation, 8 and cell proliferation. 9 However, the detailed mechanisms responsible for O2− production under different pathophysiological circumstances remain to be defined.

We have recently shown that arterial endothelin-1 (ET-1) levels are elevated in deoxycorticosterone acetate (DOCA)–salt hypertensive rats, 10 and that this resulted in increased O2− production via ET, receptor activation 10 in this low-renin hypertension model. 11 However, the mechanisms contributing to increased ET-1 are unknown. The peptide hormone 8-argininevasopressin (AVP) is able to stimulate preproendothelin-1 mRNA expression in arteries and cultured endothelial cells. 12–14 Furthermore, the enhanced endothelin gene expression observed in DOCA-salt rats was absent in vasopressin-deficient Brattleboro rats treated with DOCA-salt, 13 suggesting a functional link between vasopressin and ET-1 gene expression.

The biological effects of vasopressin are mediated through 2 AVP receptor subtypes, the V1 and V2 receptors. 15–17 V1 receptors mediate vasoconstriction, proliferation, and hypertrophy, 18,19 whereas V2 receptors are present in renal epithelial cells, where they control free water and urea reabsorption. 20,21 Chronic treatment with a vasopressin V1 receptor antagonist lowered blood pressure and attenuated the increase in preproendothelin-1 gene expression observed in mesenteric arteries of DOCA-salt rats. 14,22 It is thus likely that V1 receptors are involved in vasopressin-induced vascular ET-1 upregulation in DOCA-salt hypertension. However, the relationships among vasopressin, ET-1, and vascular O2− production are unknown. In the present study, we tested the hypothesis that vasopressin contributes to ET-1–induced vascular superoxide production in DOCA-salt hypertensive rats. Our findings indicate that arterial ET-1 production is stimulated by vasopressin via its V1 receptors, resulting in increased superoxide levels.

Methods

DOCA-Salt Hypertensive Rats

DOCA-salt hypertension was created in adult male Sprague-Dawley rats as previously described. 7 Briefly, rats (250 to 275 g, Charles...
River, Portage, Mich) were uninephrectomized (flank incision, left side) under pentobarbital (50 mg/kg IP)-induced anesthesia, and a silicone rubber DOCA implant (200 mg/kg) was placed subcutaneously between the shoulder blades. Sham rats were uninephrectomized but received no implant (control group), whereas normal rats were untreated. DOCA rats received 1% NaCl and 0.2% KCl in their drinking water. Sham and normal rats received tap water. All animals were fed standard rat chow and had ad libitum access to both food and drinking solutions. Hypertension develops gradually in this model, with arterial pressure rising steadily over a 4-week period. Blood pressure was measured using noninvasive tail-cuff measurements in conscious, but restrained and pretrained rats. Carotid arteries were collected between weeks 4 to 6 after DOCA implantation. All animal procedures were in accordance with the institutional guidelines of the Michigan State University.

Immunohistochemistry and Immunoassay for Arterial Vasopressin

Immunohistochemistry was performed as previously described. Briefly, cross sections of the vessel (6 μm thin) were fixed in ice-cold aceton for 10 minutes, and endogenous peroxidase was inhibited with 0.3% (v/v) hydrogen peroxide for 30 minutes. Sections were blocked with 5% horse serum/PBS–Tween-20 (pH 7.4) for 20 minutes and then incubated with the primary antibody at room temperature for 2 hours diluted in PBS–Tween-20 containing 2% horse serum. The primary antibody used was rabbit polyclonal antibody for AVP (1:1000, Oncogene, CN Bioscience Inc). Nonimmune rabbit IgG was used as a negative control at the same concentration as that of the primary antibody. Sections were then incubated for 30 minutes with biotinylated secondary goat anti-rabbit antibody (Oncogene), diluted at 1:200 in PBS–Tween-20 containing 2% horse serum. Visualization was performed with an AEC kit (Vector Laboratories). Nuclei were counterstained with Gill’s hematoxylin. Images were obtained through a digital camera (SPOT, Diagnostic Instruments Inc).

To quantify arterial vasopressin levels, isolated carotid arteries were cleaned and homogenized in lyses buffer (100 mmol/L K2 HPO4, 1 mmol/L PMSF, and 0.2% Triton X-100) and subjected to centrifugal concentrator under vacuum and were stored at 80°C until use. The vessel homogenates were centrifuged at 20,000 g for 15 minutes at 4°C. C18 Sep-Pak columns (100 mg, Fisher Scientific) were used for sample extraction. After extraction, samples were evaporated for dryness with a centrifugal concentrator under vacuum and were stored at −20°C. The dry samples were reconstituted with assay buffer and measured immediately with a commercial vasopressin immunoassay kit (Assay Designs Inc). Vasopressin concentrations were calculated with Prism 3.02 software (GraphPad).

Luminometric Immunoassay for Arterial ET-1 Levels

The ET-1 levels of carotid arteries were determined as described previously. Isolated carotid arteries of sham and DOCA rats were incubated for 24 hours at 37°C with or without a selective V1 vasopressin receptor antagonist (β-Mercapto-β-β-cyclopenta-methylenepropiony1, O-Me-Tyr2, Arg3 vasopressin, ME-AVP, 10−8 mol/L, Sigma), whereas arteries of normal rats were incubated with AVP (10−7 mol/L, Sigma) under the same conditions. After incubation, arteries were frozen in liquid nitrogen, homogenized for 1 minute in 1 mol/L acetic acid containing 1.5x10−5 mol/L peptatin (Sigma), and immediately boiled for 10 minutes. After being chilled, the vessel homogenates were centrifuged at 20,000 g for 30 minutes at 4°C, and the supernatants were stored at −80°C until use. The supernatants were subjected to ET-1 assay using a commercial ET-1 immunoassay kit (R&D Systems). A microplate luminometer (Fluoroskan Ascent FL, Labsystems) was used to measure the intensity of the light emitted. Ascent software 2.4.1 (Labsystems) was used for calculation of ET-1 levels.

Arterial O2− Levels

The isolated carotid arteries of sham or DOCA rats were incubated for 24 hours with or without ME-AVP at 10−6 mol/L, or incubated with a selective ET1 receptor antagonist ABT627 (3x10−6 mol/L, Abbott Laboratories) for 1 hour, whereas the arteries of normal rats were incubated with AVP at 0, 10−6, 10−5, or 10−4 mol/L for 4 or 24 hours. Some carotid arteries of normal rats were incubated for 1 hour with ABT627 (3x10−6 mol/L) or ME-AVP (10−6 mol/L) before AVP addition (10−7 mol/L for 24 hours). Vascular O2− was then assayed using both oxidative dihydroethidium (10−6 mol/L, Molecular Probes) fluorescence and lucigenin (5x10−6 mol/L, Sigma) chemiluminescence as previously described.7

Data Analysis

Data were expressed as mean±SEM. Repeated-measures ANOVA was used for comparison of multiple values obtained from the same subject, whereas factorial ANOVA was used for comparing data obtained from 2 independent groups of animals. The Bonferroni procedure was used to control type 1 errors. A value of P<0.05 was considered statistically significant.

Results

Elevated Vasopressin Levels in Carotid Arteries of DOCA Rats

There was a significant increase in systolic arterial blood pressure in DOCA-salt rats, compared with sham controls (176±4 versus 117±2 mm Hg; n=24 rats, P<0.01). Vasopressin immunoreactivity was higher in vessels from DOCA-salt rats compared with sham rats, and was mainly localized in the media (Figure 1A). The difference in arterial vasopressin levels between DOCA and sham groups was determined by immunoassay, and the results showed a 3-fold increase in arteries of DOCA-salt rats compared with sham rats (7.1±1.4 versus 1.9±0.4 pg/mg protein, DOCA versus sham rats) (Figure 1B).

Vasopressin Increases Arterial O2− Levels Via Its V1 Receptors

In carotid arteries of normal rats, vasopressin treatment for 24 hours dose-dependently increased arterial O2− level, and pretreatment of the vessels for 1 hour with ME-AVP, a selective V1 receptor antagonist, blocked vasopressin-induced O2− (Figure 2A). Vasopressin treatment for 4 hours did not increase arterial O2− levels (data not shown). In DOCA-salt rats, the increased arterial O2− levels were significantly reduced after ME-AVP treatment for 24 hours compared with levels for nontreated controls (Figure 2B).

Vasopressin Induces O2− Via Increasing Arterial ET-1 Levels

The selective ET1 receptor antagonist ABT627 abolished vasopressin-induced increments in arterial O2− in both normal (Figure 2A) and DOCA-salt rats (Figure 2B). On the other hand, vasopressin treatment for 24 hours significantly elevated arterial ET-1 levels in normal rats compared with nontreated controls (Figure 3A). Vasopressin treatment for 4 hours did not increase arterial ET-1 levels significantly (data not shown). There was a marked increase of arterial ET-1 levels in DOCA-salt rats compared with sham rats, and inhibition of V1-vasopressin receptors with ME-AVP significantly reduced the ET-1 levels (Figure 3B). The effects of ABT627 on vasopressin-induced arterial O2− in normal rats.
and arterial $O_2^-$ in DOCA-salt rats were further confirmed by oxidative dihydroethidium fluorescent confocal microscopy (Figure 4).

Discussion

The present study demonstrates, for the first time, that (1) arterial vasopressin levels are increased in carotid arteries of DOCA-salt hypertensive rats, (2) vasopressin stimulates vascular $O_2^-$ production through activation of V1 receptors, and (3) ET-1 is an intermediary signaling molecule in vasopressin-stimulated $O_2^-$ formation. These findings are consistent with our hypothesis that vasopressin stimulates arterial ET-1 production, resulting in increased superoxide levels in mineralocorticoid hypertension.

Several previous studies have suggested that vasopressin induces ET-1 gene expression and may play an important role in DOCA-salt hypertension.12–14.22.24.25. Brain and plasma levels of vasopressin mRNA and the peptide are increased in rats after DOCA-salt treatment,26.27 and V1-vasopressin receptor antagonists reduce blood pressure in DOCA-salt hypertension.22.24 However, there is no report to date on the arterial levels of vasopressin in DOCA-salt hypertension. In the present study, we demonstrate for the first time that arterial tissue vasopressin levels are $\approx$3-fold higher in DOCA-salt rats than in sham rats. The source of this increased vasopressin is likely from the circulating blood, consistent with previous published findings.26.27

In our recent studies, we have shown that arterial ET-1 levels are significantly elevated in DOCA-salt hypertensive rats,10 which contribute to the augmented $O_2^-$ levels.7.10 Because vasopressin has been shown to stimulate preproendothelin-1 mRNA expression in arteries and cultured endothelial cells,12–14 we examined the possible effect of vasopressin on arterial superoxide production. Our data indicate that vasopressin stimulated superoxide production in carotid arteries of normal rats in 24 hours, an effect that was abolished by both the V1 receptor antagonist ME-AVP and the ET$_A$ receptor antagonist ABT627. These findings suggest that vasopressin induces $O_2^-$ production via its V1 receptors, and the effect is ET-1 dependent. Similarly, the elevated arterial superoxide levels in DOCA-salt rats were blunted by both V1 receptor antagonist ME-AVP and ET$_A$ receptor

Figure 1. A, Immunohistochemical detection of vasopressin expression in the cross sections of a typical artery of sham (a and c) and DOCA-salt rat (b and d). Positive vasopressin immunoreactivity, as indicated by red staining, is mainly localized to the medium. The sections shown are typical of 3 separate experiments. Bar, 0.05 mm (a and b) or 0.1 mm (c and d). B, Vasopressin levels in carotid arteries of sham and DOCA-salt rats. Extractions of carotid arteries of sham or DOCA-salt rats were subjected to vasopressin assay using a commercial immunoassay kit. n=4 to 5 rats. *$P<0.05$ vs sham.
antagonist ABT627, suggesting that the increased arterial vasopressin stimulates O$_2^-$ production via V1 receptors in DOCA-salt hypertension. These findings are consistent with a recent study showing that vasopressin-induced hemodynamic responses in DOCA-salt hypertension are reduced by ETA receptor antagonism. 28

The direct effect of vasopressin on arterial ET-1 production was also investigated in the present study. Our results demonstrate that ET-1 levels were significantly increased in carotid arteries treated with vasopressin for 24 hours in vitro. Furthermore, the V1 receptor antagonist ME-AVP significantly reduced ET-1 levels in carotid arteries of DOCA-salt rats and had no effect on the basal arterial ET-1 levels in sham rats. Because arterial vasopressin levels are 3-fold higher in DOCA-salt rats than in sham rats, as shown in the present study, these data together suggest that vasopressin stimulates arterial ET-1 in DOCA-salt rats compared with sham controls. This conclusion is further supported by our findings that after 24-hour incubation, vasopressin significantly increased O$_2^-$ levels in carotid arteries of normal rats, in addition to augmenting ET-1 levels. Similarly, the V1 receptor antagonist ME-AVP decreased both ET-1 and O$_2^-$ levels in carotid arteries of DOCA-salt rats after 24-hour incubation. The work of others has shown that elevation in ET-1 expression
observed in DOCA-salt rats is attenuated in vasopressin-deficient Brattleboro rats \(^{13}\) or in rats treated chronically with a V1 antagonist. \(^{14,22}\) Therefore, it is likely that high arterial ET-1 levels stimulated by vasopressin contribute to increased arterial O\(_2^•\) levels in this low-renin model of hypertension. Direct functional studies are underway to verify that increased vasopressin stimulates in vivo ET-1 and superoxide production in DOCA-salt rats.

It is of interest to note that an incubation time of 24 hours was required for vasopressin to increase either ET-1 or superoxide levels in the carotid arteries of normal rats, whereas an incubation time of 4 hours failed to produce this effect. The most likely explanation for these experimental observations is that vasopressin-induced arterial superoxide production depends on the newly synthesized ET-1 peptide from preproET-1, a process that requires a period of longer than 4 hours. \(^{29,30}\) Alternatively, the cellular source for new ET-1 synthesis could be smooth muscle cells instead of endothelial cells, because agonists such as cytokines have been shown to stimulate ET-1 release quite rapidly from endothelial cells. \(^{31}\)

However, it is important to point out that although we observed that arterial vasopressin levels are \(\approx\)3-fold higher in DOCA-salt rats than in sham rats (7.1±1.4 versus 1.9±0.4 pg/mg protein), previously published studies showed that the normal plasma vasopressin levels (1 to 30 pmol/L) are generally elevated by 3- to 5-fold in the benign phase of hypertension in this model. \(^{32,33}\) In addition, although ABT627 reversed the effect of vasopressin on vascular O\(_2^•\) levels, vasopressin treatment of carotid arteries of normal rats for 24 hours in vitro only elevated arterial ET-1 levels to a small extent compared with the increase in vasopressin levels observed in the vessels of DOCA-salt rats. Similarly, MEAVP only reduced arterial ET-1 levels by \(\approx\)40% in DOCA-salt rats after 24-hour incubation. A possible reason for these experimental observations may be that the effects of vasopressin on both ET-1 and superoxide levels observed in carotid arteries of DOCA-salt rats were chronic in nature (several weeks) and at an advanced stage of hypertension (ie, 4 to 6 weeks after DOCA implantation). Nonetheless, our data are consistent with the published findings that vasopressin increases both ET-1 mRNA expression \(^{12}\) and ET-1 release\(^{34}\) in rat mesenteric arteries and that the effects of vasopressin in modulating vascular structure and function are at least in part mediated by enhanced ET-1 expression. \(^{12-14}\)

**Perspectives**

In conclusion, the present study demonstrates, for the first time, that vasopressin stimulates arterial ET-1 production via its V1 receptors, resulting in increases superoxide levels in carotid arteries of DOCA-salt hypertensive rats. These findings may provide a novel insight into the humoral mechanisms of low-renin hypertension.

**Acknowledgments**

This work was supported in part by American Heart Association Grant 0130537Z, American Diabetes Association Research Award 7-01-RA-10, and Juvenile Diabetes Research Foundation Innovative Grant 5-2001-311 (to A.F.C.).

**References**


Vasopressin Induces Vascular Superoxide Via Endothelin-1 in Mineralocorticoid Hypertension
Lixin Li, James J. Galligan, Gregory D. Fink and Alex F. Chen

Hypertension. 2003;41:663-668; originally published online December 16, 2002; doi: 10.1161/01.HYP.0000047875.43777.79
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/41/3/663

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/