Digoxin Prevents Ouabain and High Salt Intake–Induced Hypertension in Rats With Sinoaortic Denervation

Bing S. Huang, Marian Kudlac, Raj Kumarathasan, Frans H.H. Leenen

Abstract—Digoxin prevents ouabain-induced hypertension in rats. In the present study, we tested whether this effect of digoxin depends on its sensitizing effect on baroreflex function or is due to an antagonistic action on exogenous ouabain or endogenous ouabainlike activity (“ouabain”) in the brain. In Wistar rats, resting mean arterial pressure (MAP) was significantly increased by long-term subcutaneous (SC) ouabain (75 μg/d) plus high salt (8%) intake for 12 days (but not after only 5 days). In rats with chronic sinoaortic denervation (SAD), MAP was increased within 5 days of ouabain treatment to the same extent as MAP after 12 days of treatment in intact rats. The effect of ouabain and high salt was prevented when digoxin was given SC concomitantly via osmotic minipump (200 μg · kg⁻¹ · d⁻¹). Resting MAP was not changed in rats treated with digoxin alone. In a second set of rats with chronic SAD or sham surgery, high salt intake was given for 14 days, with or without SC digoxin (200 μg · kg⁻¹ · d⁻¹) or intracerebroventricular (ICV) antibody Fab fragments (200 μg/d), which bind “ouabain” with high affinity. On day 14, MAP, central venous pressure, heart rate, and renal sympathetic nerve activity were recorded in conscious rats at rest and in response to air-jet stress, IV phenylephrine and nitroprusside, and acute volume expansion with 5% dextrose IV. In rats with SAD versus sham surgery, high salt significantly increased resting MAP as well as excitatory responses of MAP, heart rate, and renal sympathetic nerve activity to air stress. These effects of high salt in rats with SAD were prevented by digoxin or Fab fragments. Arterial baroreflex function was blunted but cardiopulmonary baroreflex function was not affected in rats with SAD. Digoxin and Fab fragments had no effects on either function. In an in vitro assay for the inhibitory effects on Na⁺,K⁺-ATPase activity, 20 ng of ouabain caused 29% inhibition, but 20 ng of ouabain plus 13 or 53 ng of digoxin caused only 16% or 4% inhibition, respectively. These data indicate that the arterial baroreflex opposes sympathoexcitatory responses to ouabain and “ouabain” in the brain, thereby delaying ouabain- and preventing high salt–induced hypertension in Wistar rats. In addition to possible effects on the arterial baroreflex, digoxin appears to act centrally to prevent the sympathoexcitatory and pressor effects of increased brain “ouabain” or ouabain.

Key Words: digoxin ■ nerve activity, sympathetic, renal ■ sodium intake ■ baroreflex ■ ouabain ■ denervation, sinoaortic ■ stress, air ■ Fab

High salt intake increases endogenous compounds with ouabainlike activity (“ouabain”) in the brain of rats with salt-sensitive blood pressure (BP) and to a lesser extent in the brain of normotensive rats with salt-insensitive BP. 1,2 In salt-sensitive rats, such as spontaneously hypertensive and Dahl salt-sensitive rats, the increase in brain “ouabain” appears to mediate salt-induced sympathetic hyperactivity, impairment of baroreflex function, and hypertension. 3,4 In normotensive rats, high salt intake sensitizes arterial baroreflex control of sympathetic activity 1 and does not cause sympathetic hyperactivity and hypertension. 3,5 High salt increases BP after functional removal of the arterial baroreflex by sinoaortic denervation (SAD), 7–9 which suggests that sensitization of the arterial baroreflex prevents hypertension in intact normotensive rats with high salt intake. Therefore it is possible that the increase in brain “ouabain” by high salt in normotensive rats 1,2 leads to sympathetic hyperactivity but that this stimulatory effect is offset by an inhibitory effect from the sensitized baroreflex.

Sensitization of arterial baroreceptors may also be crucial for the time course of the development of exogenous ouabain–induced hypertension. Long-term peripheral administration of ouabain leads to sympathoexcitation 10 and hypertension, 10–12 even in intact normotensive rats. However, the onset of the hypertension shows a delay of several days. 10,12 Peripherally administered ouabain can sensitize arterial baroreceptors. 13 This sensitization may be responsible for temporarily offsetting central pressor effects of ouabain, thereby leading to the delay of ouabain-induced hypertension. Digoxin is structurally similar to ouabain but has been reported to prevent ouabain-induced hypertension in normo-
tensive rats. Like ouabain, digoxin also can sensitize arterial baroreflex function, which may facilitate a decrease in sympathetic outflow and blood pressure. In humans with congestive heart failure, long-term administration of digoxin decreases sympathetic activity. However, intravenous (IV) injection of digoxin had no effects on baroreflex function but did lower sympathetic activity in patients with right ventricular failure. Thus, it appears that a sympathoinhibitory effect of digoxin may not only result from sensitization of baroreflex function. We postulated that the sympathoinhibitory effect of digoxin depends not only on baroreflex sensitization but also on its central actions; ie, digoxin may act as a partial agonist, and in the presence of high exogenous ouabain or endogenous “ouabain,” as in salt-sensitive hypertension and congestive heart failure, it acts as an antagonist of ouabain in the brain.

Hence, the objectives of the present study are 2-fold: (1) to test the hypothesis that increases in exogenous ouabain or endogenous “ouabain” in the central nervous system through long-term subcutaneous (SC) administration of ouabain and/or high salt intake play a functional sympathoexcitatory and pressor role in Wistar rats but that sensitization of arterial baroreflex function delays or prevents the development of sympathetic hyperactivity and hypertension, and (2) to test in these 2 models of “ouabain”- and ouabain-induced hypertension whether long-term administration of digoxin can prevent sympathetic hyperactivity and hypertension due to a possible antagonistic action on exogenous ouabain or endogenous “ouabain” in the brain.

Methods

The present study was performed in accordance with the guidelines of the University of Ottawa Animal Care Committee. Male Wistar rats (7 to 8 weeks old, 200 to 250 g) were purchased from Charles River Breeding Laboratories, Montreal, Canada, and housed at constant room temperature, humidity, and light (12-hour light/dark cycle). Body weight was followed for all study groups and showed only minor differences (data not shown).

After 3 to 5 days of adaptation, each rat received SAD or sham surgery, as described previously. Briefly, rats were anesthetized with an injection of sodium pentobarbital (50 mg/kg IP) mixed with atropine (0.4 mg/kg). The neurovascular sheaths enclosing common carotid arteries, vagi, and sympathetic trunks were exposed through a midline incision at the neck. SAD was completed by sectioning bilaterally the sheaths, cervical sympathetic trunks, superior laryngeal nerves, and aortic depressor nerves and by stripping the area of carotid bifurcation and painting the region with 10% phenol in ethanol. Sham surgery consisted of exposure of the sheaths and cervical sympathetic trunks, superior laryngeal nerves, and aortic depressor nerves and by stripping the area of carotid bifurcation and painting the region with 10% phenol in ethanol. Sham surgery was performed for 4 hours, and then the catheter was connected to a transducer; BP and heart rate (HR) were recorded through a polygraph (model 7E, Grass Instrument Co) and a Grass 7P44 tachograph. Resting MAP and HR were recorded for 10 minutes, after a 30-minute stabilization period.

Blockade of Ouabain-Induced Hypertension With Digoxin

This study was done in SAD rats only. One week after SAD, under halothane anesthesia, rats were implanted SC with either 3 ouabain or placebo pellets respectively, as described above. In half of the rats, osmotic minipumps (model 2 ML2; rate, 5 μL/h; Alzet Corp) filled with digoxin (200 μg · kg⁻¹ · d⁻¹, Eli Lilly Canada Inc) were also implanted SC into the back of each rat. The dose of digoxin was based on the original studies by Manunta et al. Four groups of rats were involved in this protocol: rats treated with SAD and placebo (n=6); SAD and placebo, and digoxin (n=6); SAD and ouabain (n=5); and SAD, ouabain, and digoxin (n=6). High salt diet was started after pellet implantation. Twelve days after the start of high salt intake, rats were anesthetized with halothane and catheters were placed into the right femoral artery and vein. Resting hemodynamics and responses to phenylephrine were recorded, as described in the above protocol.

Blockade of High Salt-Induced Hypertension With Digoxin and Fab Fragments

To test blockade of high salt–induced hypertension with digoxin and Fab fragments, rats with SAD or sham surgery were used. One week after the surgery, 2 groups of rats with SAD and 1 group of sham rats were given halothane inhalation, and a 23-gauge stainless steel right-angled cannula was implanted into the left lateral ventricle, as previously described. The cannula was connected to an osmotic minipump (model 2002; rate, 12 μL/d; Alzet) for long-term intracerebroventricular (ICV) infusion of antibody Fab fragments (Digidink, Glaxo Wellcome Inc) or γ-globulins (Sigma Chemical Co) as control (200 μg/d for both). In another group of rats with SAD, minipumps (model 2 ML2; rate, 5 μL/h) filled with digoxin (200 μg · kg⁻¹ · d⁻¹, Eli Lilly Canada) were implanted SC. Rats were divided into 4 groups: those given sham surgery and γ-globulins, SAD and γ-globulins, SAD and Fab fragments, and SAD and digoxin. After the surgery, all rats were provided with high salt for 2 weeks.

At the end of dietary period, each rat was given halothane anesthesia in the early morning, and catheters were placed into a femoral artery and vein and into the right jugular vein and advanced to the level of the right atrium. Each rat was given methohexital sodium (Brevital 30 mg/kg IV supplemented with 10 mg/kg as needed; Eli Lilly Canada Inc), and through a flank incision, a pair of silver electrodes (A-M System, Inc) was placed around and fixed to the left renal nerve with silicone rubber (SilGil 604, Wacker). At least 4 hours after recovery from the anesthesia, the rat was placed in a testing cage that permitted movement back and forth. The intrarterial catheter and the catheter in the jugular vein were connected to a transducer, and BP, HR, and central venous pressure (CVP) were recorded as described above. The electrodes were linked to a Grass P511 bandpass amplifier, and renal sympathetic nerve activity (RSNA; spikes per second) was counted by a nerve traffic analyzer (model 706C, University of Iowa Bioengineering) and digitalized. The RSNA was determined by subtracting noise from the total activity.

After a 30-minute stabilization period, basal MAP, HR, CVP, and RSNA were recorded. To assess sympathetic responsiveness, a standardized air stress was then provided for 30 seconds twice at...
10-minute intervals using an air stream (1 to 1.5 PSI) directed to the face of the rat. Twenty minutes after the responses to air stress had subsided, phenylephrine was infused IV as described above. Ten minutes after return to baseline, nitroprusside was infused (5 to 100 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) IV) to induce a ramp MAP decrease with maximum of −50 mm Hg for 1 to 2 minutes. After a 30-minute rest, acute volume expansion was performed twice with IV infusion of 2 doses of 5% dextrose solution (3.3 and 10.0 mL/kg of body weight over 30 seconds) at 5-minute intervals.

The in vitro assay for the estimation of the inhibitory effect of ouabain and digoxin on Na\(^+\)K\(^-\)ATPase was described previously. Quantification was done by measuring \( ^{32}P \) liberation from [\( \gamma ^{32}P \)]ATP (New England Nuclear) that was hydrolyzed by ouabain-sensitive Na\(^+\)K\(^-\)ATPase prepared from dog kidney (Sigma Chemical Co) in or not in the presence of specific amounts of ouabain and digoxin. The assay was performed 3 times. Samples were measured in quadruplicate, and the mean values were entered for analysis.

**Statistical Analysis**

For the protocol of salt-induced hypertension, phenylephrine-induced decreases in HR and increases in MAP at 5-mm Hg increments were analyzed as a linear model, and the arterial baroreflex function was estimated by the gain (slope) of their linear relation. For the protocol of salt-induced hypertension, responses of RSNA were expressed as percent of baseline, and changes in both RSNA and HR were analyzed as a linear model, and the arterial baroreflex gain was estimated by the gain (slope) of their linear relation. The in vitro assay for the estimation of the inhibitory effect of ouabain and digoxin on Na\(^+\)K\(^-\)ATPase was described previously. Quantification was done by measuring \( ^{32}P \) liberation from [\( \gamma ^{32}P \)]ATP (New England Nuclear) that was hydrolyzed by ouabain-sensitive Na\(^+\)K\(^-\)ATPase prepared from dog kidney (Sigma Chemical Co) in or not in the presence of specific amounts of ouabain and digoxin. The assay was performed 3 times. Samples were measured in quadruplicate, and the mean values were entered for analysis.

**Results**

**Ouabain-Induced Hypertension**

**Treatment for 5 Days**

Ouabain treatment combined with high salt for 5 days did not increase resting MAP in sham rats. In rats with SAD, high salt alone for 5 days also did not increase MAP. In contrast, in rats with SAD on high salt, ouabain significantly increased MAP compared with the other 3 control groups. HR was similar among the 4 groups of rats (Table 1).

**Treatment for 10 to 12 Days**

In SAD rats on high salt alone for 10 to 12 days, MAP showed a minor (not significant) increase. In sham rats treated with ouabain plus high salt for 10 to 12 days, resting MAP was significantly increased, to an extent similar to that observed in the rats with SAD given the same treatment for 5 days only. In rats with SAD treated with ouabain plus high salt, resting MAP was increased to the same extent after 10 to 12 days as after 5 days. Resting HR was similar among the 4 groups of rats. In sham rats, activation of the arterial baroreflex by IV phenylephrine caused the expected reflex decreases in HR. This reflex was blunted in rats with SAD, as reflected by markedly reduced baroreflex gains. Ouabain treatment did not affect the reflex function in rats with SAD but increased baroreflex gain significantly in sham rats (Table 1).

**Blockade of Ouabain-Induced Hypertension by Digoxin in Rats With SAD**

Treatment with digoxin alone did not change MAP, whereas treatment with ouabain alone significantly increased MAP. When ouabain was combined with digoxin, the increase in MAP by ouabain was prevented. In all 4 groups of rats, arterial baroreflex control of HR was blunted, and treatment with ouabain, digoxin, or both in combination did not affect the reflex function (Table 1).

**Blockade of Salt-Induced Hypertension**

After 14 days of high salt intake, MAP was significantly higher in rats treated with SAD and \( \gamma \)-globulins versus those treated with sham surgery and \( \gamma \)-globulins (Table 2).
contrast, in SAD rats treated with either ICV Fab fragments or SC digoxin, resting MAP did not increase and remained similar to that in the sham rats. There were no significant differences in HR and CVP among the 4 groups of rats (Table 2).

Responses to Air Stress
Air stress rapidly increased RSNA, MAP, and HR (Figure). In SAD rats on high salt treated with γ-globulins, the extent of increases in RSNA, MAP, and HR was twice that in sham rats on high salt. These enhanced responses were similarly prevented by either ICV Fab fragments or digoxin. When the responses were expressed as a percentage of their resting values, the responses changed in patterns similar to those observed for absolute values (data not shown).

Arterial Baroreflex
In SAD versus sham rats treated with γ-globulins, the baroreflex control of either RSNA or HR was blunted, with markedly reduced gains. These changes in SAD rats treated with γ-globulins remained when Fab fragments or digoxin were given ICV (Table 2).

Cardiopulmonary Baroreflex
Volume expansion increased CVP and decreased RSNA and HR. The maximum increase in MAP was <3 mm Hg in all groups of rats. The gains in baroreflex control for either RSNA or HR were similar in the 3 groups with SAD and tended (not significantly) to be higher versus sham rats (Table 2).

In Vitro Na⁺,K⁺-ATPase Inhibitory Activity
Ouabain 20 ng caused a 31±1% inhibition of Na⁺,K⁺-ATPase activity. Digoxin alone (13 or 53 ng) caused 13±2% or 35±5% inhibition. Digoxin prevented the inhibitory effects of ouabain in a dose-related manner: 16±2% and 4±1% inhibition of Na⁺,K⁺-ATPase activity by 20 ng ouabain plus 13 and 53 ng of digoxin, respectively.

Discussion
The present study shows several new findings. First, in intact Wistar rats, high salt intake plus long-term SC administration of ouabain for 10 to 12 (but not 5) days increases resting BP; in rats with chronic SAD, by 5 days, this treatment increased BP to the same extent as by 10 to 12 days in intact rats. Second, in the absence of a functional arterial baroreflex, digoxin prevents hypertension induced by long-term ouabain in rats with SAD and on high salt intake. Third, high salt intake causes sympathetic hyperactivity and hypertension in Wistar rats with chronic SAD, and both can be prevented by antibody Fab fragments administered ICV or by SC digoxin.

Baroreflex Sensitization and High Salt Intake (Endogenous “Ouabain”)–Induced Hypertension
In normotensive rats with salt-insensitive BP, high salt intake causes sensitization of baroreflex function and does not increase BP. Hypertension develops after 1 to 2 weeks when chronic SAD is combined with high salt intake. In Sprague-Dawley rats with SAD, MAP started to increase significantly after 11 days of high salt intake. In Wistar-Kyoto rats with SAD, high salt for 4 weeks significantly increased MAP. The present study shows that in Wistar rats with SAD, high salt diet tends to increase MAP at day 10 to 12 and significantly increases MAP at day 14. Although high salt intake increases brain “ouabain” in normotensive rats, a functional role for brain “ouabain” in causing sympathoexcitation (as it does in salt-sensitive rats) so far has not been demonstrated. The present study demonstrates that, in contrast to results in intact rats, in rats with SAD, high salt intake for 2 weeks not only increases resting BP, but also enhances sympathoexcitatory and pressor responses to air stress. These

![Graph](http://hyper.ahajournals.org/)
effects of high salt can be prevented by blockade of brain “ouabain” with Fab fragments. This suggests that in normotensive rats, high salt–induced increases in brain “ouabain” play a functional role in increasing sympathetic activity and BP; however, these effects normally are masked by sensitization of the arterial baroreflex and can be unmasked and fully expressed after chronic SAD. Because high salt also increases plasma “ouabain,” it is possible that peripheral “ouabain” contributes to the arterial baroreflex sensitization (ie, in intact normotensive rats on high salt intake, sympathoexcitatory effects caused by increased “ouabain” in the brain are compensated for by sensitization of baroreflex function by higher plasma “ouabain” levels).

**Baroreflex Sensitization and Exogenous Ouabain–Induced Hypertension**

Peripheral administration of ouabain for 10 days via pellets (25 to 75 μg/d) significantly increased BP and brain content of ouabain in Wistar rats. The hypertension can be prevented by long-term treatment with ICV Fab fragments or reversed by acute ganglionic blockade by IV hexamethonium. Thus, in addition to possible peripheral mechanisms, peripherally administered ouabain appears to act centrally to increase sympathetic outflow and BP. Ouabain administered IV (10 μg/d) caused hypertension on day 12 but not day 10. When 30 μg·kg⁻¹·d⁻¹ of ouabain was infused SC in Sprague-Dawley rats a latency of 7 to 14 days was observed before BP increased significantly. In the present study, in intact Wistar rats, ouabain pellets (75 μg/d) plus high salt intake did not cause hypertension by day 5, but did by day 10 to 12. Thus, the onset of hypertension by peripheral ouabain occurs with a delay of several days. In rats with chronic SAD, the same treatment for only 5 days increased BP to the same extent as in intact rats treated with ouabain for 12 days. Consistent with a previous study, the present study shows that, in Wistar rats, ouabain administered peripherally sensitizes arterial baroreflex function. Thus, in rats treated long term with ouabain, it appears that, sensitization of the arterial baroreflex temporarily inhibits central sympathoexcitatory and pressor effects of ouabain and that the central effects of ouabain at the present dose prevail after a few days of delay.

It is not clear at present why in intact normotensive rats ouabain alone induces hypertension but high salt intake alone does not. Differing extents of increase in brain ouabain may be one causal mechanism. In Wistar-Kyoto rats, high salt for 4 weeks increased hypothalamic “ouabain” <1-fold without increasing BP. However, in Wistar rats, treatment with ICV, IV, or SC ouabain (10 or 25 μg/d) for 10 to 14 days increased hypothalamic “ouabain” ≈3-fold with a significant increase in resting BP. In Sprague-Dawley rats, administration of ouabain 30 μg·kg⁻¹·d⁻¹ SC for 5 weeks also increased hypothalamic “ouabain” ≈3-fold with an increase in BP. Thus, in normotensive rats versus rats given long-term treatment with ouabain, high salt intake alone does not seem to increase brain “ouabain” to levels that are high enough to overcome the inhibitory effects of peripheral “ouabain”-induced baroreflex sensitization.

**Blockade of Ouabain- or “Ouabain”-Induced Hypertension by Digoxin**

In contrast to ouabain, digoxin does not cause hypertension in rats with SAD. Manunta et al reported that digoxin 100 μg·kg⁻¹·d⁻¹ SC for 12 days inhibited ouabain-induced hypertension in Sprague-Dawley rats without decreasing peripheral ouabain levels. In the present study, digoxin prevented both ouabain- and high salt–induced hypertension in rats with SAD. Moreover, in rats with SAD and on high salt, both ICV Fab fragments and SC digoxin prevented enhancement in sympathoexcitatory and pressor responses to air stress, as well as the development of hypertension. Because peripherally administered digoxin can enter the central nervous system readily because of its lipophilic properties, digoxin appears to be able to act centrally as an antagonist for brain “ouabain” just as do Fab fragments.

Digoxin may antagonize the sympathoexcitatory and pressor responses to brain “ouabain” or ouabain through several mechanisms. Although both ouabain and digoxin are recognized as inhibitors of Na⁺,K⁺-ATPase, different binding sites were reported for digoxin and ouabain on sodium pumps of cultured proximal tubular cells of dog kidney, which can distinguish between ouabain and digoxin. Thus, with different lipophilicities, which determine how readily the compounds cross the blood-brain barrier, ouabain and digoxin may reach and bind different areas in the brain, causing either sympathoexcitation or sympathoinhibition. Also, such compounds as canrenone, a major metabolic product of spironolactone, have been shown to act as a partial agonist at the ouabain receptor site of Na⁺,K⁺-ATPase and to exert an antagonistic effect against higher concentrations of ouabain. We therefore speculate that digoxin may act as a partial agonist for the Na⁺,K⁺-ATPase, with lower “intrinsic” activity than ouabain (ie, digoxin may be an antagonist in the presence of high exogenous or endogenous ouabain). Indeed, in an in vitro study, digoxin inhibited the enzyme (Na⁺,K⁺-ATPase)-inhibiting activity of ouabain in a dose-related fashion. However, the enzyme was obtained from dog kidney, and species- and tissue-dependent differences exist in the sensitivity to ouabain and digoxin and in the enzyme α1-isof orm distribution.

In summary, the present study establishes that arterial baroreflex sensitization represents an important compensatory mechanism in Wistar rats to blunt or prevent hypertension induced by long-term ouabain and/or high dietary salt. In normotensive rats, an increase in brain “ouabain” by long-term high salt intake appears to play a sympathoexcitatory and pressor role. However, sensitization of arterial baroreflex function, possibly by increased plasma “ouabain,” may offset the effects of brain “ouabain” and prevent the development of hypertension. In contrast, in rats treated with ouabain long term, the sensitized arterial baroreflex only delays the hypertension. In both models characterized by increased ouabain or “ouabain” in the brain, SC digoxin can prevent the development of sympathetic hyperactivity and hypertension by acting centrally as an antagonist for brain ouabain or “ouabain.”

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