The Peripheral Action of Clonidine Analog ST-91: Involvement of Atrial Natriuretic Factor

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ABSTRACT

It is generally thought that the cardiovascular and renal effects of clonidine, an \alpha-2 adrenergic agonist, are mediated by central mechanisms. Our previous work has shown that diuresis and natriuresis caused by central administration of clonidine are mediated by an enhanced release of atrial natriuretic factor (ANF). Because clonidine has been shown to have peripheral actions the objective of the present study was to determine whether ANF is also involved in these actions. Studies were performed with use of a structural clonidine analog, ST-91, which does not cross the blood-brain barrier. Intravenous injection of various doses (0–250 \mu g/rat) of ST-91 into conscious, normally hydrated female Sprague-Dawley rats (200–250 g) produced dose-related increases in urinary output, which were accompanied by significant increases in urinary sodium, potassium and cGMP excretion. Compared with saline, the highest dose of ST-91 (250 \mu g/rat) during the first hour of treatment significantly (P < .001, n = 18) enhanced urinary output (0.2 ± 0.1 vs. 3.0 ± 1.1 ml/h) and excretion of sodium (28 ± 4 vs. 345 ± 50 \mu mol/h), potassium (10 ± 4 vs. 165 ± 37 \mu mol/h) and cGMP (191 ± 29 vs. 1340 ± 322 pmol/h), the biological marker of ANF. These renal responses were associated with increased plasma ANF (59 ± 7 vs. 810 ± 28 pg/ml, P < .001, n = 12), measured 10 min after ST-91 (250 \mu g/rat), which remained elevated for at least 1 h (P < .01, n = 6). The enhanced renal responses that were induced by 10 \mu g ST-91 were partially, yet significantly inhibited by yohimbine (50 \mu g), an \alpha-2 antagonist. On the other hand, efaroxan (500 \mu g), an \alpha-2 adrenergic receptor antagonist, showed a stronger inhibitory effect, whereas naloxone (0.8 mg) had no effect. Pretreatment of rats with anti-ANF reduced the diuretic and natriuretic effects of ST-91. These results indicate that the renal effects of ST-91 are mediated by imidazole as well as by \alpha-2 adrenergic receptors, but not by opioid receptors. Furthermore, the renal effects evoked by ST-91 are mediated by ANF.

An acute administration of the \alpha-2 adrenergic agonist, clonidine, an imidazoline derivative usually used as an antihypertensive drug, produces diuresis and diuresis in experimental animals and humans (Gehr et al., 1986; Schmitt, 1977). It has been suggested that these renal effects and the antihypertensive action are caused by the activation of centrally located \alpha-2 adrenoceptors (Kobinger, 1978; Schmitt and Schmitt, 1970). There is also evidence that central opioid receptors may also be involved in centrally induced renal and cardiovascular effects of clonidine (Farsang and Kunos, 1979; Mastrianni and Ingenito, 1987). However, recent studies showed that the antihypertensive effect of clonidine is mediated through stimulation of another class of receptors that are distinct from \alpha-2 adrenoceptors. Clonidine binds with high affinity to imidazole binding sites in the rostral ventrolateral medulla oblongata (Michel and Insel, 1989; Molderings et al., 1991).

The diuretic effect of clonidine is mediated by ANF (Pan and Gutkowska, 1988), a potent diuretic, natriuretic and vasorelaxant hormone (De Bold et al., 1981; Ballermann and Brenner, 1985). We have demonstrated that this effect is caused by the activation of central opioid receptors (Pan and Gutkowska, 1988). The medulla oblongata has been suggested as a site of hypotensive and bradycardic action of clonidine (Kobinger, 1978; Laubie and Schmitt, 1977). Although a central effect of the \alpha-2 adrenergic agonist, clonidine, is generally accepted, there is also evidence that clonidine has peripheral effects. Significant antinociception was promoted by a clonidine analog, which does not cross the blood-brain barrier (Blandford and Smyth, 1989; Nakamura and Ferreira, 1988). Imidazoline receptors have also been shown to be present in peripheral tissues such as bovine adrenal medulla and rabbit kidney (Wang et al., 1992; Limon et al., 1992). These observations prompted us to investigate whether clonidine exerts its cardioendocrine effects through peripheral mechanisms that involve ANF.

Consequently, we used the clonidine structural analog, ST-91, with \alpha-2 adrenergic activities, which does not

ABBREVIATIONS: ANF, atrial natriuretic factor; ST-91, 2-(2,6-diethylphenylamino)-2-imidazoline hydrochloride.

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cross the blood-brain barrier (Kobinger and Pichler, 1975). Experiments were undertaken to characterize the effects of ST-91 on water and electrolyte excretion in conscious, normally hydrated rats with use of various doses of ST-91; and also to determine whether the cardiovascular and renal effects of ST-91 are mediated by ANF.

Methods

Experimental protocol. Experiments were performed on conscious, normally hydrated female Sprague-Dawley rats weighing 200 to 250 g. The animals were housed four per cage at 22°C under 12-h light/dark cycle (room illumination from 6:00 A.M. to 6:00 P.M.). Purina laboratory chow (Ralston-Purina, St. Louis, MO) and water were available ad libitum before the experiment.

The effect of different doses of ST-91 on water and electrolyte excretion was evaluated in 18 conscious, normally hydrated rats per group. All experiments began at 9:00 A.M. The rats were placed in restraint cages for 1 to 2 min and injected into the tail vein with different doses of ST-91 (0, 1, 10, 100 and 250 μg) dissolved in 0.9% saline. After the injection, the rats were placed individually in metabolic cages without food and water. Urine volume was measured with a calibrated syringe every hour for 4 consecutive hours. The rats were returned to their cages at the end of the experiment.

Other experiments were similarly performed on groups of 12 rats pretreated with either naloxone (0.8 mg/300 μl saline), yohimbine (50 μg/300 μl saline) or efaroxan (500 μg/300 μl saline) injected into the tail vein 10 min before ST-91 (10 μg) administration. The dose of efaroxan used was determined in preliminary experiments as the lowest dose that inhibited the diuretic effect of 10 μg of ST-91. Two doses, 50 and 100 μg of yohimbine, were used, but the higher concentrations of yohimbine did not inhibit the responses further. The dose of naloxone is double what has been shown to counteract the effects of ST-91.

The implication of ANF in the renal effects of ST-91 was determined by pretreatment of six rats with 0.4 ml of anti-ANF serum injected intravenously into the tail vein 10 min before ST-91 (100 μg) injection. The control animals in this group received 0.4 ml of normal rabbit serum.

Rat anti-ANF serum was used to determine the specificity of the ST-91 effect on urine output and electrolyte excretion. The anti-serum was produced in New Zealand white rabbits by immunization with the antigen obtained by coupling 26 amino acid carboxyterminal peptide of ANF to bovine serum albumin (Hamet et al., 1989). The antibody is specific for C-terminal ANF (Gutkowska, 1987). This antibody is specific for C-terminal ANF peptides. The cross-reactivity with Ser99-Tyr126 circulating peptide is 100%; the antibody also recognizes the 126-amino acid prohormone Asn1-Tyr126.

Blood pressure was recorded on conscious rats by the tail-cuff method. Pressure was measured at −5 and at 10, 20, and 40 min posttreatment with 1, 2.5 and 10 μg of ST-91. Plasma ANF was determined in blood samples obtained from decapitated rats 10 min after the administration of various doses (1–100 μg) of ST-91, and at 15, 30 and 60 min posttreatment with 10 μg ST-91. Two milliliters of blood were collected in chilled tubes containing the protease inhibitors: 1 mg ethylenediaminetetraacetic acid, 10 μl of 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO; no. P-7626) and 10 μl of 0.5 mM pepstatin A (Sigma, no. P-4265) per 1 ml of blood, then centrifuged for 10 min at 3000 rpm at 4°C. Plasma ANF was assayed by radioimmunoassay (Gutkowska, 1987) after prior extraction by heat-activated Vycor glass beads (Gutkowska et al., 1984).

Urinary cGMP excretion was measured by radioimmunoassay according to a previously described method (Hamet et al., 1989). Urinary sodium and potassium concentrations were measured with a flame photometer (Perkin-Elmer 51, Norwalk, CT), and excretions per hour were calculated.

Materials. ST-91 hydrochloride (kindly provided by Boehringer Ingelheim Laboratories, Ontario, Canada), yohimbine hydrochloride (Sigma, no. Y3125), naloxone hydrochloride (Dupont Canada NEN, Mississauga, Ontario, no. 1241) and efaroxan (Sigma, no. E3263) were prepared in 0.9% sterile saline immediately before the injection.

Statistical analysis. Data storage, graphical output and statistical analysis assessed by two-way analysis of variance were accomplished with RS1 data analysis software (BBN, Cambridge, MA). Statistical significance was taken as P < .05. All data are reported as means ± S.E.M.

Results

Intravenous injection of ST-91 to conscious, normally hydrated rats evoked a dose-related increase in urine output during 4 h of treatment. Cumulative urine output over 4 h increased by 7-fold with the dose of 250 μg of ST-91 as compared with values obtained in control animals (1.2 vs. 8.4 ml/h, P < .001, n = 18) (fig. 1). However, the most prominent effect was observed during the first hour (fig. 2), in which a 15-fold increase in urine volume was noted with 250 μg of ST-91 (0.2 ± 0.1 vs. 3.0 ± 1.1 ml/h, P < .001, n = 18).

Figure 2 also shows that urinary sodium excretion increased by 12-fold from 28 ± 4 μmol/h in control rats to 401 ± 37 μmol/h (P < .001) with the dose of 100 μg of ST-91 and plateaued with 250 μg (344 ± 49 μmol/h). Similarly, potassium excretion increased during the first hour after ST-91 injection (250 μg) from 10 ± 4 to 165 ± 37 μmol/h (P < .001) (fig. 2).

Urinary cGMP, an index of ANF activity, was measured in experimental and control groups of animals during the first hour after ST-91 administration. Urinary cGMP was augmented in a dose-related manner, from 191 ± 21 pmol/h in control animals, to 1340 ± 322 pmol/h (n = 18, P < .001)
during the first hour after ST-91 (100 μg) administration (fig. 2).

These renal effects paralleled the significant 14-fold increase in plasma ANF measured 10 min after administration of 100 μg ST-91 (59 ± 7 vs. 810 ± 28 pg/ml, P < .001, n = 12) (fig. 3). Moreover, compared with rats that received only saline injections, 10 μg ST-91 resulted in elevated plasma ANF levels, and this elevation was sustained during 1-h posttreatment (41 ± 1 vs. 115 ± 15 pg/ml, P < .01, n = 6).

The possible hemodynamic effects of ST-91 such as an increase in blood pressure which could induce ANF release are unlikely. Blood pressure measurement in conscious rats treated with three different doses of ST-91 (1, 2.5 and 10 μg) revealed mild dose-dependent reduction at 10 min after treatment (fig. 4). Blood pressure returned to basal levels at 20 min with the lower doses of ST-91, but remained at sub-basal levels (122.4 ± 3.7 vs. 109.6 ± 5.7 and 113.1 ± 4.5 mm Hg) at 20 and 40 min, respectively, when the highest dose (10 μg) was used.

The implication of the peripheral opioid receptors in the action of ST-91 was tested by using naloxone. When the animals were pretreated with the opioid antagonist naloxone at relatively high dose of 0.8 mg/rat, 10 min before ST-91 (10 μg) administration, the increase in blood pressure was still present (fig. 5).
administration, no alteration in urine output or sodium and potassium excretion was observed (fig. 5), which indicated that the opioid receptors are not involved in the peripheral action. On the other hand, the involvement of alpha-2 adrenergic receptors in the enhanced urinary output and electrolyte excretion in response to ST-91 was determined with use of the alpha-2 adrenergic antagonist yohimbine. Intravenous pretreatment with yohimbine (50 μg) 10 min before ST-91 (10 μg) partially, yet significantly blocked the diuresis, natriuresis and kaliuresis (fig. 5) that were evoked by ST-91 administration. However, efaroxan, an imidazoline receptor antagonist, inhibited the renal responses to ST-91 (10 μg) administration most effectively (fig. 5), which implies that it is mainly the peripheral imidazoline receptors that are involved in the renal responses to ST-91. Furthermore, the ST-91- stimulated excretion of cGMP was inhibited by efaroxan but not by yohimbine or naloxone (fig. 5), suggesting that ANF may be involved in imidazoline but not alpha-2 adrenergic receptor-mediated actions.

Pretreatment with anti-ANF serum (0.4 ml) 10 min before ST-91 (100 μg) administration did not affect sodium excretion but significantly (more than 50% inhibition, P < .05) blocked the ST-91-induced diuresis and natriuresis during
stimulation of centrally located vagal tone, both effects being attributed to the inhibiting the sympathetic nervous activity, concomitant with chloride, is an antihypertensive drug believed to act by inhibition of the renal effects of ST-91, which was blocked by cGMP, by peripheral injections of the clonidine analog, ST-91.

The opioid antagonist, naloxone, showed no effect on anti-ANF; and c) the inhibition of the renal effects of ST-91 increased urine output induced by ST-91, which was blocked by peripheral mechanisms and that ANF is an element in the neuronal pathway in the brain, clonidine may act on the brainstem (Kunos et al., 1981) and the corticotrophs of the anterior pituitary (Vale et al., 1978) to release beta-endorphins which may further contribute to the antihypertensive action of clonidine. The hypotensive effect of clonidine is inhibited by intracerebroventricular administration of beta-endorphin antiserum (Ramirez-Gonzalez et al., 1983; Naranjo et al., 1985).

A general consensus has been reached that the brainstem is the site of cardiovascular action of clonidine, specifically in the nucleus tractus solitarius or ventrolateral medulla. Clonidine promotes analgesia (Paalzow and Paalzow, 1976; Fielding et al., 1978) through both supraspinal (Paalzow, 1974) and spinal levels (Hare and Franz, 1983), and blocks neurophysiological and behavioral symptoms of opioid withdrawal (Aghajanian, 1978; Gold et al., 1978) by central mechanisms. However, analgesia is also promoted by a structural analog of clonidine, ST-91, which does not cross the blood-brain barrier (Bentley et al., 1977). ST-91 is more potent than clonidine in stimulating postjunctional alpha adrenoceptors (Kobinger and Pichler, 1975) and is equipotent with clonidine in stimulating presynaptic inhibitory alpha adrenoceptors (Scriabine et al., 1977). Nakamura and Ferreira (1988) tested ST-91 on hyperalgesia induced by intralplanar injection of prostaglandin E₂ or carrageenin. The antinoceptive effect of ST-91 was dose-dependent, possibly mediated by enkephalin-like substances and indicated peripheral actions.

We and others have previously shown that ANF, whose main source is the cardiac atria, is involved in the cardiovascular effects of clonidine (Baranowska et al., 1987a,b, 1988; Pan and Gutkowska, 1988; Ferrari and Agnoletti, 1989); and that opioids are important stimuli of ANF release, findings confirmed by others (Horky et al., 1985; Gutkowska et al., 1986; Crum and Brown, 1988; Chen et al., 1989; Ogutman et al., 1990). Therefore, we hypothesized that the structural clonidine analog, ST-91, may, by direct effect on the heart or by indirect mechanism via opioids, induce the release of ANF. In fact, the results of the present study show that the diuretic and natriuretic effects of ST-91 were evoked by peripheral mechanisms and that ANF is an element in the cascade of events. However, the opioid receptors are not involved in these effects. It is important to note that the present studies were performed on conscious, normally hydrated animals in which the effect of anesthesia, which may alter the responses to the drugs, was eliminated. Microinjection of clonidine into nucleus tractus solitarius produced pressor responses in conscious animals (Kubo and Misu, 1981) contrary to depressor effect in anesthetized animals (Vlahakos et al., 1985).

The enhanced renal responses to ST-91 were not blocked by naloxone, indicating that peripheral opioids are not a part of the mechanisms of action of the clonidine analog, ST-91. This is compatible with several studies in normotensive humans (Watkins et al., 1980; Pedrinelli et al., 1985) and animals (Farsang et al., 1980; Elghozi et al., 1981; Shropshire and Wendt, 1983) where naloxone does not antagonize the accumulated evidence that the antihypertensive action of clonidine is also caused by the involvement of the central opioid receptors (Farsang and Kunos, 1979; Mastrianni and Ingenito, 1987), because the opioid antagonist naloxone attenuates the hypotensive effects of clonidine in spontaneously hypertensive rats (Naranjo et al., 1985). In addition to its function in the neuronal pathway in the brain, clonidine may act on the brainstem (Kunos et al., 1981) and the corticotrophs of the anterior pituitary (Vale et al., 1978) to release beta-endorphins which may further contribute to the antihypertensive action of clonidine. The hypotensive effect of clonidine is inhibited by intracerebroventricular administration of beta-endorphin antiserum (Ramirez-Gonzalez et al., 1983; Naranjo et al., 1985).

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The present studies provide strong evidence that the renal responses evoked by the peripheral actions of ST-91, a structural analog of clonidine with alpha-2 adrenergic activities, are mainly mediated by the imidazoline receptors and partially by adrenergic receptors through the stimulation of ANF release. These findings were demonstrated by a) the dose-dependent enhancement of diuresis, natriuresis and kaliuresis, as well as plasma ANF and its biological marker cGMP, by peripheral injections of the clonidine analog, ST-91, which does not cross the blood-brain barrier; b) the increased urine output induced by ST-91, which was blocked by anti-ANF; and c) the inhibition of the renal effects of ST-91 by efavoxan which was associated with reduced cGMP excretion. The opioid antagonist, naloxone, showed no effect on diuresis or natriuresis induced by ST-91.

Clonidine, 2-(2,6-dichlorophenylamino)-2 imidazole hydrochloride, is an antihypertensive drug believed to act by inhibiting the sympathetic nervous activity, concomitant with an increase in vagal tone, both effects being attributed to the stimulation of centrally located alpha-2 adrenoceptors (Onesti et al., 1969; Schmitt and Schmitt, 1970). Further studies

Discussion

![Fig. 6. Urinary output and sodium excretion induced by ST-91 (100 μg) with or without pretreatment with anti-ANF serum (0.4 ml) 10 min before administration of ST-91. *P < .05 vs. ST-91 alone (n = 6 each).](image-url)
effect of clonidine. Moreover, the lack of naloxone effect observed in our study is in agreement with Vollmar et al. (1987) and Mosqueda-Garcia and Kunos (1988), who suggested that peripherally located opioid receptors are unlikely to be involved in the cardiovascular effects of clonidine or ANF release.

We are proposing a new peripheral mechanism for the diuresis and natriuresis induced by the clonidine analog, ST-91, namely that the renal effects are caused by an enhanced release of ANF that is independent of systemic hemodynamic changes. The mechanisms that mediate the renal actions of ANF may include increased glomerular filtration rate, inhibition of distal sodium reabsorption or medullary washout. The relative importance of these mechanisms is beyond the scope of this study. However, to get some insight into the mechanisms by which ST-91 enhances ANF release, we used efaroxan, an imidazoline receptor antagonist. Indeed, our studies show that both the peripheral alpha-2 adrenoceptors and, more importantly, peripheral imidazoline receptors are activated by ST-91, because the renal effects are mainly inhibited by efaroxan and partly by yohimbine. Studies carried out on isolated atri (Nishimura et al., 1990; Garcia et al., 1986; Gibbs, 1987), atrial cardiocytes (Gibbs, 1987) or in intact animals (Rankin et al., 1987) show that beta adrenoceptors promote the release of ANF. Whether specific imidazoline receptors are found in the heart (Fuder and Schwarz, 1993) and whether the activation of these receptors or the heart alpha-2 adrenoceptors (Fuder and Schwarz, 1993; Starke et al., 1989) could be responsible for the direct release of ANF has to be determined, especially that ANF is synthesized and released from other organs (Gutkowska and Nemer, 1989).

Interestingly, ST-91 induced an important increase in urinary cGMP excretion which supports the role of activated ANF system in the renal responses. The enhanced excretion of cGMP was blocked by efaroxan. Yohimbine tended to decrease urinary cGMP, but naloxone had no effect. Therefore, these findings suggest that it is mainly the imidazoline receptors that are involved in ANF release and subsequent renal responses to ST-91. However, the role of alpha-2 adrenoceptors can not be ruled out. Molecular cloning showed at least three subtypes of alpha-2 adrenergic receptors in rats, only two of which exhibit a high affinity to yohimbine (Harrison et al., 1991; Uhlen and Wikberg, 1991). In summary, we propose that ST-91, a clonidine analog with partial alpha-2 adrenergic properties, acts peripherally on the renal system to enhance diuresis and natriuresis. These actions may be mediated by the ST-91-induced release of plasma ANF, through activation of peripheral imidazoline receptors, independently of peripheral opioid receptors.

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References


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