

Full Paper

Effects of AF-DX116 and Other Muscarinic Receptor Antagonists on Orthostatic Hypotension in Autonomic Imbalanced (SART-Stressed) Rats

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Received October 25, 2004; Accepted January 14, 2005

Abstract. SART (specific alternation of rhythm in temperature)-stressed rats are an animal model of autonomic imbalance created by exposing animals to repeated cold stress. The SART-stressed rats have been shown to easily develop orthostatic hypotension (OH). In this study, effects of AF-DX116, a selective M₂ antagonist, and other muscarinic receptor antagonists on OH were investigated in SART-stressed and unstressed rats. Each anesthetized rat was cannulated into the left common carotid artery, and blood pressure (BP) and heart rate were measured. Stimulation for postural change was initiated by head-up tilting. As the indices of OH, the maximum fall of BP, % reflex (recovery from maximum fall), and the area enclosed between the baseline and the recovery curve for BP (AUC) were used. Large AUC and small % reflex in SART-stressed rats were changed, becoming similar to those of the unstressed rats by AF-DX116 and methoctramine. Atropine and methylatropine had similar effects to AF-DX116. However, the effects of methoctramine, atropine, and methylatropine were less than that of AF-DX116. Pirenzepine was not effective. In conclusion, it was suggested in SART-stressed rats that OH was related to hyperactivity in the parasympathetic nerve and the M₂ receptor played the major role in OH.

Keywords: SART (specific alternation of rhythm in temperature) stress, AF-DX116, orthostatic hypotension, muscarinic receptor, head-up tilt

Introduction

Orthostatic hypotension (OH) (1), which is a manifestation of insufficient neural control of circulation, is a cause of transient cerebral ischemia (2) and may induce neurally-mediated syncope (3). OH, which is induced by autonomic dysfunction, stress, and other factors, often occurs in hypotensive people, the elderly with decreased biodynamics, and epileptic people who are highly sensitive to the influences of the external environment.

Many people complain of feeling unwell when air temperature changes drastically or when seasons change such as in early spring and autumn. Animals loaded with SART (specific alternation of rhythm in temperature) stress (4) have symptoms of autonomic imbalance.

SART-stressed animals are created by exposing animals to cold temperature repeatedly, in short, environmental temperature is altered repeatedly and rapidly between room temperature and low temperature. SART-stressed animals have also autonomic imbalance of the parasympathetic hypertonicity-sympathetic hypotonicity type (5). Abnormalities reported in SART-stressed animals include the cardiovascular system. In conscious SART-stressed rats, resting blood pressure (BP) is lower than that of unstressed rats and hypotension is continuous (6). Reported also are abnormal electrocardiographic changes and increased heart rate (HR) in SART-stressed mice/rats (7–10). In addition, our recent studies found that the SART-stressed rats had marked OH, suggesting the rats could be an OH model animal (9). In order to elucidate the mechanism of OH, we investigated the effect of AF-DX116, a selective muscarinic M₂-receptor antagonist, which has been observed to remarkably

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improve hypotension in SART-stressed rats (10). Effects of other muscarinic receptor antagonists were investigated and compared with that of AF-DX116. OH was evoked by the head-up tilt test (11), which is widely used to test autonomic functions and neurally-mediated reflex.

Materials and Methods

Experimental animals and procedure for SART stress loading

Experimental animals: Male Wistar rats (Japan SLC, Inc., Hamamatsu) aged 8–10 weeks and weighing 250–300 g at the start of the study, were used in accordance with ethical procedures following the guidelines for the care and use of laboratory animals issued by the Japanese government and The Japanese Pharmacological Society. The animals were housed in groups of three in a wire-net cage (38 × 25 × 17 cm) placed in a temperature- and light-controlled room (24 ± 1°C; with a 12-h light-dark cycle, lights on at 08:00, off at 20:00) with free access to a standard diet (MF; Oriental Yeast, Tokyo) and tap water ad libitum.

Procedure for SART stress loading: According to procedures reported previously (12), three rats per group were alternately transferred to two cages, one of which was placed in a room at 24°C and the other in a room at –3°C, every hour from 09:00 to 16:00 and housed in a cage at –3°C from 16:00 to 09:00 the following morning. This procedure was repeated for 6–8 days up to 11:00 on the day of the experiment. The stressed rats were kept at room temperature (24°C) for at least 30 min before the experiment to avoid the direct influence of the cold environment.

Measurement of blood pressure and heart rate

These measurements were conducted according to our previous report (9). Each rat was anesthetized with urethane (1.2 g/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA), with additional administration of 1/10th of the initial dose, when necessary for maintaining anesthesia. Each rat was restrained on a board in a supine position. The pressure transducers were connected to polyethylene catheters (0.965 mm i.d., PE-50; Becton, Dickinson and Company, NJ, USA) which were inserted into the left common carotid arteries. For monitoring BP, pulse waves were recorded on a portable pen recorder (305711; Yokogawa, Tokyo) via strain amplifiers (AS 1202; NEC, Tokyo) continuously, from 3 min before to 8 min after raising the board. HR was recorded by using an electrocardiograph (ECG 5201; Nihon Kohden, Tokyo) through lead II. We injected heparin (0.38 mL/h of 10 units/mL solution, Heparin sodium

injection; Takeda Chemical Industries, Ltd., Osaka) to prevent blood coagulation in cannula. The rats were kept at about 37°C with a thermoregulator (Nippon Medical & Chemical Instrument Co., Ltd., Osaka).

Systolic blood pressure (SBP) was measured from the pulse waves before tilting and at 0 min (immediately after), 30 s, 1 min, and every one minute. ECG was recorded before tilting and at 0 min (immediately after), 30 s, 1 min, 2 min, and 4 min after tilting the board and 3 min after returning to the horizontal position; HR (beats/min) was calculated by using the number of R waves on the ECG chart for 2 s.

Stimulation for postural change (head-up tilt)

According to procedures reported previously (9), postural change was initiated by rapidly raising the head part of the board from the horizontal supine position to a 60° head-up tilt position. This condition was maintained for 4 min, after which the board was returned to the original horizontal position.

Three parameters were used for indicating the degree of OH. The tilting indices are 1) the maximum fall of BP caused by the head-up tilt, 2) % reflex obtained from the reflection (the maximum increase from the lowest level of the decreased BP within 2 min from the start of tilting), and 3) the area under the curve, AUC, enclosed between the baseline and the recovery curve for BP from 0 to 4 min (mmHg · min).

BP and ECG were monitored and measured, from 3 min before to 5 min after tilting for 4 min.

Drugs

AF-DX 116 (Boehringer Ingerheim Japan, Kawanishi), a selective M₂-receptor antagonist; atropine monosulfate (Wako Pure Chemical Industries, Ltd., Osaka) and atropine methylbromide (methylatropine), nonselective M receptor antagonists; pirenzepine dihydrochloride (Sigma Chemical Co.), a selective M₁ receptor antagonist; and methoctramine tetrahydrochloride, a selective M₂ receptor antagonist (Sigma Chemical Co.) were used.

AF-DX 116 was dissolved in 0.1 N HCl at 0.1 mL, the solution was neutralized by 0.1 N NaOH, adjusted to pH 7.4, and then diluted with saline just before use. Other drugs were dissolved with 0.9% physiological saline and administered at a volume of 0.1 mL/100 g wt. These drugs were administered intravenously or orally at 60 min before head-up tilting. Rats of the orally administered group were fasted during 2 h before the experiment and had free access to water.

Evaluation of drug effect

The effect of drugs on % reflex and AUC were

calculated by the following formula:

$$\text{Effect (\%)} = \frac{(\text{Values before drugs in stressed rats} - \text{Values after drugs in stressed rats})}{(\text{Mean values before drugs in stressed rats} - \text{Mean values before drugs in unstressed rats})} \times 100$$

Statistical analyses

Data obtained from 4–8 rats/group are expressed as means \pm S.E.M. and statistically analyzed by an unpaired Student's *t*-test for data from two groups and by one-way or two-way analysis of variance (ANOVA) and Tukey's test for data from multiple groups. Significance was set at $P < 0.05$.

Results

Effects of AF-DX116

Figure 1 shows the effects of AF-DX116 on the % reflex, which indicates degrees of recovery from OH induced by postural changes; following intravenous administration (i.v.) (Fig. 1a) and following oral administration (p.o.) (Fig. 1b).

In unstressed rats in the 100 $\mu\text{g/kg}$, i.v., 200 $\mu\text{g/kg}$, i.v., 12 mg/kg, p.o., and 24 mg/kg, p.o. group, the % reflex prior to AF-DX116 administration was $67.5 \pm 5.1\%$, $70.6 \pm 4.8\%$, $68.5 \pm 7.6\%$, and $62.3 \pm 9.9\%$, respectively, and hardly changed following either intravenous or oral administration of the agent at the doses shown. In the SART-stressed groups, the % reflex prior to administration was $36.3 \pm 4.1\%$, $31.9 \pm 3.1\%$, $27.2 \pm$

5.7%, or $16.1 \pm 6.9\%$, respectively, significantly smaller than that in the respective unstressed group. Following either intravenous or oral administration of AF-DX116, the % reflex increased to 57.1 ± 6.6 , 59.0 ± 3.6 , 59.3 ± 8.0 or 46.3 ± 7.3 , respectively, demonstrating marked improvement in the SART-stressed rats.

AF-DX116 had no significant effect on the maximum fall of BP in either the unstressed group or SART-stressed group (data not shown). In the experiments, the SBP of unstressed and SART-stressed rats in the resting state before drugs were 118.8 ± 1.4 and 98.3 ± 1.7 mmHg for the intravenous administration groups and 119.0 ± 1.1 and 101.5 ± 2.0 mmHg for the oral administration groups, respectively. The maximum fall of BP in unstressed and SART-stressed rats in the resting state before drugs were 19.6 ± 1.2 and 23.0 ± 0.9 mmHg for the intravenous administration groups and 17.9 ± 1.4 and 31.2 ± 1.4 mmHg for the oral administration groups, respectively.

Figure 2 shows the effects of AF-DX116 on AUC. As regards to AUC, there was hardly any change in the unstressed group before and after either intravenous or oral AF-DX116 administration. In the SART-stressed group, the AUC prior to administration was between 59.5 ± 4.0 and 107.3 ± 6.1 mmHg \cdot min, which was significantly larger ($P < 0.01$) than that of the unstressed group: 34.8 ± 1.5 to 40.6 ± 5.1 mmHg \cdot min. Following intravenous administration of 100 and 200 $\mu\text{g/kg}$ of AF-DX116, the AUC in SART-stressed rats decreased. Similarly, following oral administration of 12 and 24 mg/kg of AF-DX116, the AUC in SART-stressed rats decreased markedly, while no change occurred at these doses in the unstressed group.

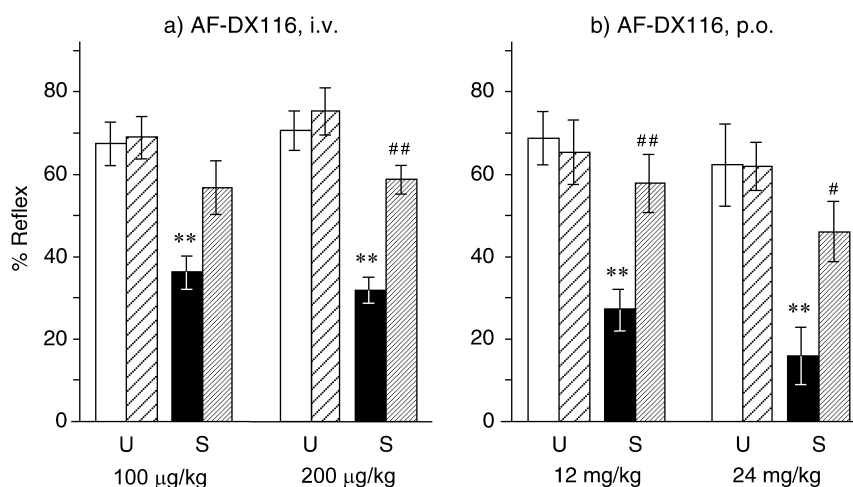


Fig. 1. Effect of AF-DX116 on % changes of blood pressure reflex induced by head-up tilting in rats. Open and closed columns: Control before AF-DX 116; striped and hatched columns: Drug-treated group. U, Unstress; S, SART stress. Data show the mean \pm S.E.M. from 6–8 rats/group. ** $P < 0.01$ vs the respective unstressed group; # $P < 0.05$, ## $P < 0.01$ vs the respective control group.

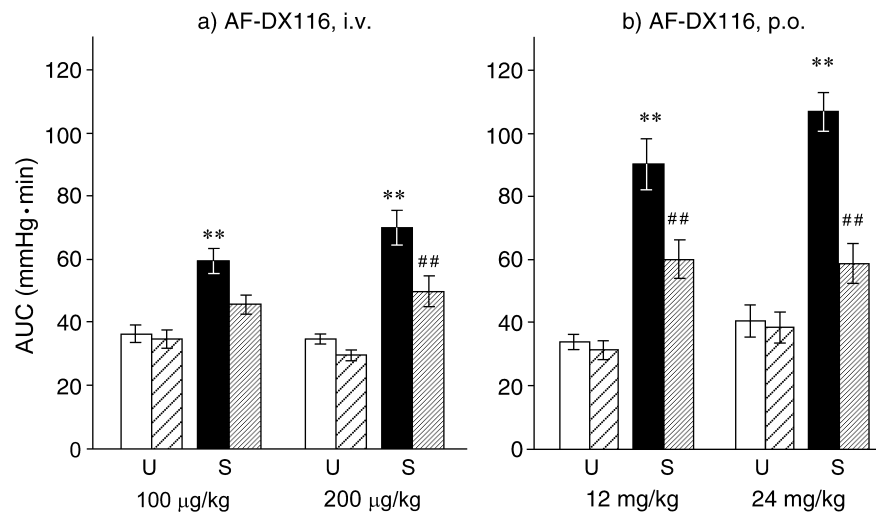


Fig. 2. Effect of AF-DX116 on AUC of blood pressure induced by head-up tilting in rats. Open and closed columns: Control before AF-DX 116; striped and hatched columns: Drug-treated group. U, Unstressed; S, SART stress. Data show the mean \pm S.E.M. from 6–8 rats/group. ** $P < 0.01$ vs the respective unstressed group; ## $P < 0.01$ vs the respective control group.

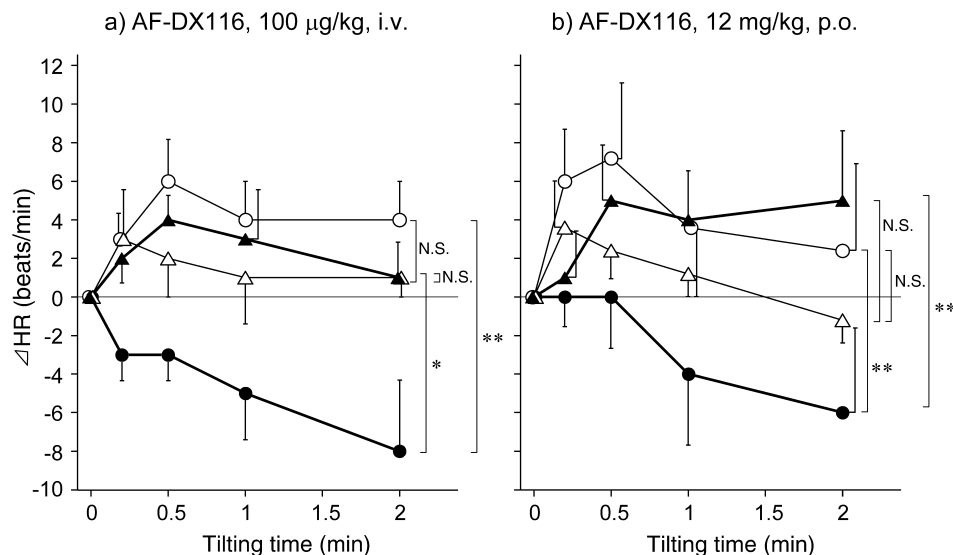


Fig. 3. Effect of AF-DX116 on heart rate changed by tilting in rats. Open and closed circles: Unstressed and SART-stressed rats, respectively; open and closed triangles: Unstressed- and SART-stressed-dosed rats, respectively. Data show the mean \pm S.E.M. from 5–7 rats/group. * $P < 0.05$, ** $P < 0.01$.

Figure 3 shows the effects of AF-DX116 on changes in HR induced by postural change stimulation. In the unstressed control group, HR increased on the head-up tilt and continued to increase for 2 min, peaking at 30 s. Following intravenous administration of 100 µg/kg of AF-DX116, the increase in HR at the head-up tilt was somewhat suppressed but not significantly. Oral administration of 12 mg/kg of AF-DX116 produced similar outcomes. In the stressed control group, HR hardly increased on the head-up tilt and was either

unchanged or decreased. Following intravenous administration of 100 µg/kg or oral administration of 12 mg/kg, decreases in HR on the head-up tilt changed to an increase in HR as seen in the unstressed group.

The above findings about the effects of AF-DX116 on the head-up tilt are summarized in Fig. 4. Following administration of AF-DX116, either intravenously or orally, in the SART-stressed rats, all indices, a smaller % reflex, larger AUC, and HR that tended to decrease, were found to be improved, while in the unstressed rats,

	Reflex		AUC		Change of Heart rate	
	Unstress	SART stress	Unstress	SART stress	Unstress	SART stress
AF-DX116, i.v.	→	↗	→	↘	→	↗
AF-DX116, p.o.	→	↗	→	↘	→	↗

- ↗ partial inhibiting-effects of AF-DX116 on decrease caused by SART stress
↘ complete inhibiting-effects of AF-DX116 on decrease caused by SART stress
↖ partial inhibiting-effect of AF-DX116 on increase caused by SART stress
→ no effect

Fig. 4. Schematic effects of AF-DX116 on appearance of orthostatic hypotension in SART-stressed rats.

Table 1. Change in AUC of blood pressure caused by muscarinic antagonists in tilting in rats

Drugs	Decrease (%)	
	Unstress	SART-stress
AF-DX116, 12 mg/kg, p.o.	8.0 ± 3.2	35.1 ± 3.0**
AF-DX116, 24 mg/kg, p.o.	3.8 ± 3.5	45.0 ± 5.4**
AF-DX116, 100 µg/kg, i.v.	5.3 ± 2.0	22.7 ± 4.1**
AF-DX116, 200 µg/kg, i.v.	15.4 ± 1.5	29.3 ± 2.9**
Methoctramine, 100 µg/kg, i.v.	11.2 ± 5.2	7.1 ± 3.2
Methoctramine, 200 µg/kg, i.v.	-31.2 ± 4.9	19.5 ± 7.2**
Pirenzepine, 400 µg/kg, i.v.	-21.3 ± 4.1	-4.7 ± 2.9**
Atropine, 40 µg/kg, i.v.	0.2 ± 6.1	19.9 ± 3.0*
Methylatropine, 500 µg/kg, i.v.	0.03 ± 2.5	26.0 ± 8.0*

$$\text{Decrease (\%)} = \frac{\text{Values before drugs} - \text{Values after drugs}}{\text{Values before drugs}} \times 100.$$

Data show the mean ± S.E.M. from 4–8 rats/group. * $P < 0.05$, ** $P < 0.01$ vs the respective normal group.

these indices were not affected at all.

Effects of other muscarinic receptor antagonists

Changes in AUC induced by various kinds of muscarinic antagonists are shown in Table 1. The changes are expressed as % decrease, which is the ratio of values after drugs minus before drugs, to values before drugs. Methoctramine showed dose-dependent decrease in AUC in the SART-stressed rats. Pirenzepine had no decreasing effect on the SART-stressed rats and increased the AUC in the unstressed rats. Atropine and methylatropine decreased the AUC in the SART-stressed rats without any effect on the unstressed rats, similarly to AF-DX116. Most of these drugs caused larger decrease in SART-stressed rats than in unstressed

Table 2. Effects of muscarinic antagonists on tilting parameters in SART-stressed rats

Drugs	Effect (%)	
	Reflex	AUC
AF-DX116, 12 mg/kg, p.o.	77.7 ± 5.9	56.8 ± 6.3
AF-DX116, 24 mg/kg, p.o.	65.3 ± 23.8	72.7 ± 10.1
AF-DX116, 100 µg/kg, i.v.	66.6 ± 14.8	59.7 ± 11.4
AF-DX116, 200 µg/kg, i.v.	70.0 ± 8.8	57.4 ± 6.6
Methoctramine, 100 µg/kg, i.v.	55.6 ± 56.1	16.3 ± 7.1
Methoctramine, 200 µg/kg, i.v.	76.4 ± 27.1	46.6 ± 15.8
Pirenzepine, 400 µg/kg, i.v.	3.3 ± 10.2	-15.0 ± 8.3
Atropine, 40 µg/kg, i.v.	35.4 ± 41.7	77.1 ± 16.4
Methylatropine, 500 µg/kg, i.v.	42.5 ± 12.4	55.3 ± 16.4

$$\text{Effect (\%)} = \frac{(\text{Values before drugs in stressed rats} - \text{Values after drugs in stressed rats})}{(\text{Mean values before drugs in stressed rats} - \text{Mean values before drugs in unstressed rats})} \times 100.$$

Data show the mean ± S.E.M. from 4–8 rats/group.

rats.

Table 2 shows the effects of muscarinic receptor antagonists including AF-DX116 on the % reflex and AUC in the SART-stressed rats. These effects are expressed as % effect.

Methoctramine improved markedly the % reflex and improved dose-dependently the AUC, similarly to AF-DX116. Pirenzepine had no effect. Atropine improved markedly the % reflex and AUC. Similarly, methylatropine improved the % reflex and AUC.

Discussion

OH is associated with symptoms such as dizziness, vertigo, and cerebral ischemia and autonomic symptoms such as loss of appetite, malaise, and headache. Most animal models of OH are created by drugs such as ganglion blockers (13, 14) and α -blockers (15, 16). The SART-stressed rats that we used in the current study have autonomic imbalance and OH with pathophysiological changes by stress (9).

In the present study, drug efficacies were evaluated based on marked hypotension that occurs immediately after standing up, % reflex (maximal recovery of BP by reflex mechanism to counteract the hypotension) and AUC as a comprehensive index of OH.

The maximum fall of BP in the head-up tilt is larger in SART-stressed rats than in unstressed rats (9). AF-DX116 had hardly any effect on the maximum fall of BP, suggesting that in SART-stressed rats that had a marked fall of BP, blood vessels of the lower extremities

were in the relaxed state, pooling large amounts of blood. AF-DX116 was markedly effective in increasing the % reflex in SART-stressed rats but not in unstressed rats. The evidence may indicate that the M_2 receptor plays a major role in OH of SART-stressed rats. It has been suggested that AF-DX116 inhibited the sympathetic suppression by acetylcholine by way of M_2 receptors that exist in the endings of sympathetic nerves that control arteries of the lower half of the body (17). Compensatory increases in HR due to the blocking of M_2 receptors may have played a role in increases in % reflex by AF-DX116. SART-stressed rats are in a state of tachycardia (9, 10) and plasma norepinephrine level was increased in SART-stressed rats (18, 19). Consequently, head-up tilting may not induce further increases in HR. Blocking of M_2 receptors at this stage inhibits vagal actions, suppressing the decreases in HR that occurs in the head-up tilt. AUC of the BP response to tilting was decreased following administration of AF-DX116 in the SART-stressed rats. The decrease of AUC at the head-up tilt seems to be contributed by increases in % reflex and HR. In Fig. 2, each control value differs in the four SART-stressed groups. Such difference in control values is considered to result from the following evidence. BP response to head-up tilt in rats has been classified into four types (9). In unstressed rats, the BP dropped rapidly by the tilt; and then tended to recover (Type A). In SART-stressed rats, the other three types of BP response were shown as follows: 1) the recovery of the BP decreased by tilting was smaller than that of the type A, 2) the decreased BP increased slightly and then gradually decreased in the upright position, 3) the decreased BP remained at the lowest level in the tilting position. Any of these three types may occur in a particular experiment. Thus the control values change in SART-stressed rats.

Following administration of pirenzepine, a selective M_1 -receptor antagonist, the OH in SART-stressed rats was not improved, and the AUC in the unstressed rats was increased. Therefore, an abnormality may also exist in the M_1 receptors in SART-stressed rats. However, the details need to be studied further.

Methoctramine caused larger AUC in unstressed rats than AF-DX116. This is possibly due to it having a higher affinity to the M_1 receptor than AF-DX116.

Atropine, which has blocking actions against both M_1 and M_2 receptors, improved OH in the SART-stressed rats. However, the changes were smaller when compared with AF-DX116. It was thought to be caused by atropine having two opposing actions, that is, through the M_2 -receptor-blocking action like AF-DX116 and also the M_1 -receptor-blocking effect like pirenzepine. Methylatropine had an effect similar to, but weaker than

atropine. AF-DX116 showed greater effects than methoctramine, possibly due to its central action (20). Atropine had greater effects due to its central action than methylatropine. It is suggested that both central and peripheral M_2 receptors play roles in OH. In the central nervous system, M_2 receptors in the cerebrum plays a role in the cardiovascular control at the head-up tilt load (21, 22). Application of M_2 -receptor agonists to the hypothalamus and medulla oblongata and the nucleus tractus solitarius located in the dorsomedial portion of the medulla oblongata induced marked hypotension with bradycardia (23). Thus, it is inferred that some abnormalities occur in both central and peripheral M_2 receptors in SART-stressed rats.

Extensive sympathetic dysfunction induces typical and severe OH (24). As described above, OH often is discussed in association with the sympathetic nerve. It was reported that SART-stressed animals were in the condition of sympathetic hypertonicity of the circulatory system (7–10) and also systemic parasympathetic hypertonicity (5). Marked OH occurred in these rats (9). Therefore, autonomic imbalance is strongly suggested in SART-stressed animals.

Postural change-induced hypotension is compensated by activation of the sympathetic nerve, that is, stimulation of α_1 - and β_1 -receptors and activation of the renin-angiotensin system through baroreflexes may also be involved in the response (25). Further studies are needed on the central and peripheral regulatory systems involved in OH in SART-stressed rats.

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