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# Mechanisms of methyclothiazide-induced inhibition of contractile responses in rat aorta

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**Abstract**

Methyclothiazide (MCTZ), a thiazide diuretic, inhibits the contractile response induced by norepinephrine in aortic rings from 12-week-old spontaneously hypertensive rats (SHR). Although not modified by indomethacin, this inhibition was attenuated by either mechanical removal of the endothelium or *N*ω-nitro-L-arginine (NOLA) treatment. These results suggest that the MCTZ effects on the norepinephrine-evoked vascular response are mediated by an endothelium-dependent mechanism involving endothelium-dependent relaxing factor (EDRF)/nitric oxide (NO) release. MCTZ was also found to alter the contractile response induced by the addition of $Ca^{2+}$ to a depolarizing solution, and this inhibitory effect was partially abolished by NOLA application. Our data led us to propose that MCTZ relaxes aortic rings, resulting in an endothelium-dependent relaxation phenomenon that could even be reinforced under high-$K^+$ depolarizing conditions. 

*Keywords:* Thiazide; Methyclothiazide; Endothelium; $Ca^{2+}$; Spontaneously hypertensive, rat (SHR); Hypertension

## 1. Introduction

Thiazide diuretics effectively lower blood pressure and reduce the risk of stroke and other cardiovascular events (Collins et al., 1990). However, the mechanisms underlying their blood pressure-reducing effect have not yet been fully elucidated. Hemodynamically, diuretics primarily decrease blood pressure by reducing extracellular fluid volume and cardiac output. Diuretics have also been shown to increase both plasma and urinary catecholamines by reflex sympathetic activation in response to excessive loss of salt and water. This sympathetic-mediated compensatory effect increases peripheral vascular resistance (Lake et al., 1979; Schiffl et al., 1981) and limits the salt and water blood pressure reduction. With long-term thiazide treatment, the plasma volume returns to baseline values and peripheral resistance decreases, suggesting a direct vascular action in addition to the diuretic effect (Van Brummelen et al., 1980). Several studies have demonstrated a direct vascular action of the thiazide diuretic, hydrochlorothiazide, and the thiazide-like diuretic, indapamide (Finch et al., 1977; Calder et al., 1992). Methyclothiazide (MCTZ), a substituted benzothiadiazide, has been found to be an orally effective diuretic substance and blood pressure lowering agent (Ford, 1960; Talso and Carballo, 1963), but its in vitro direct vascular effect has never been investigated. This study examined the direct vascular action of MCTZ and attempted to clarify its relaxation mechanism, taking into account the endothelium-dependent mechanisms involved in the action of many vasodilator compounds. These compounds mostly interact with the endothelium to cause the release of relaxing factors such as endothelium-dependent relaxing factors (EDRF)/nitric oxide (NO) and prostacyclins (Furchgott, 1984; Palmer et al., 1987). The first part of the study was a comparison of MCTZ-evoked relaxation in the presence or absence of endothelium. The effect of inhibition of NO and cyclooxygenase pathway was subsequently investigated on MCTZ-induced vasodilatation of rat aortic rings. Finally, as thiazide diuretics have been implicated in negative regulation of calcium channel activity (Pickkers and Hughes, 1995; Calder et al., 1993; Del Rio et al., 1993), and in view of the key role played by

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$Ca^{2+}$ in regulating vascular tone, we examined the possible interactions between MCTZ and $Ca^{2+}$ channels.

## 2. Materials and methods

### 2.1. Isolated tissue preparation

Thoracic aortas were obtained from 12-week-old male spontaneously hypertensive rats (SHR) and their normotensive counterparts (WKY) (Janvier, France). After anesthesia with sodium pentobarbital (50 mg/kg body weight intraperitoneally), the rats were killed by exsanguination from the common carotid arteries. The descending thoracic aorta was excised, placed in cold modified Krebs–Henseleit bicarbonate solution (composition in mM: NaCl = 115; KCl = 4.7; $MgSO_4$ = 1.2; $KH_2PO_4$ = 1.2; $CaCl_2$ = 2.5; $NaHCO_3$ = 25.0 and glucose = 11.1, pH = 7.4), and dissected free from fat and adhering connective tissues. The blood vessels were cut into rings (2–3 mm); special care was taken not to damage the luminal surface of the preparations. In some rings, the endothelium was removed mechanically.

### 2.2. Measurement of tension

The isolated rings were suspended in an organ bath containing 10 ml of Krebs–Henseleit solution at 37°C and constantly aerated with 95% $O_2$–5% $CO_2$. Two tungsten wires were inserted into the lumen, and the preparations were mounted with one tungsten wire in the organ bath, while the other tungsten wire was connected to a force-displacement transducer so that the change in tension could be measured isometrically. The segment was then stretched progressively to a passive tension of 3 g, which corresponded to the optimal point of their length–active tension relationship. Following a 40-min equilibration period and prior to the beginning of the experiments, vessel viability was assessed by exposing arteries to 60 mM KCl. Vessels which did not reproducibly produce a tension equivalent to more than 1 g in response to this stimulant were discarded. Removal of endothelium was checked by demonstrating the absence of relaxation in response to carbamylcholine ($10^{-5}$ M) during submaximal norepinephrine-induced ($10^{-7}$ M) contractions. This single dose of carbamylcholine was also given to test whether the endothelium was still intact.

Aortic rings with and without endothelium were contracted with increasing cumulative concentrations of norepinephrine ($10^{-9}$–$10^{-5}$ M) to generate concentration–response curves. The response to norepinephrine was examined in parallel rings, which had been incubated for 15 min with MCTZ ($10^{-6}$, $10^{-5}$, and $10^{-4}$ M) or its solvent (control).

In the experiments with inhibitors, intact aortic rings were incubated with the vasorelaxant drug ($10^{-4}$ M) and *N*ω-nitro-L-arginine (NOLA) ($10^{-4}$ M) or indomethacin ($10^{-7}$ M) for 15 min before cumulative addition of norepinephrine. MCTZ and NOLA were added simultaneously.

A separate series of experiments tested the effect of MCTZ on the sensitivity of the contractile machinery to $Ca^{2+}$. After equilibration with normal Krebs solution, the aortic rings with endothelium were incubated for 30 min in a high-$K^+$, $Ca^{2+}$-free medium. The rings were then incubated for 15 min with appropriate drugs and concentration–response curves were obtained by cumulatively adding $CaCl_2$ ($10^{-4}$–$10^{-2}$ M). For evaluation of the results, each curve obtained in the presence of the drug was compared with the solvent-control curve.

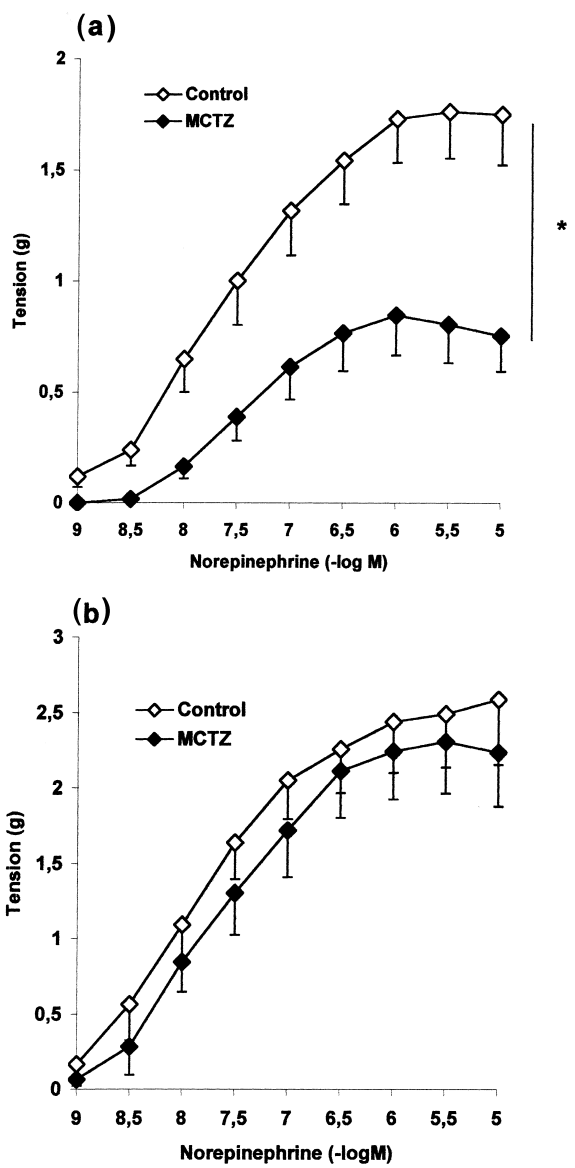


Fig. 1. Contractions in response to norepinephrine ($10^{-9}$–$10^{-5}$ M) in SHR aortic rings, with (a) and without (b) endothelium, in the absence (control) or presence of methyclothiazide (MCTZ, $10^{-4}$ M) ($n = 13$ for each group) ($^{*}P < 0.01$).

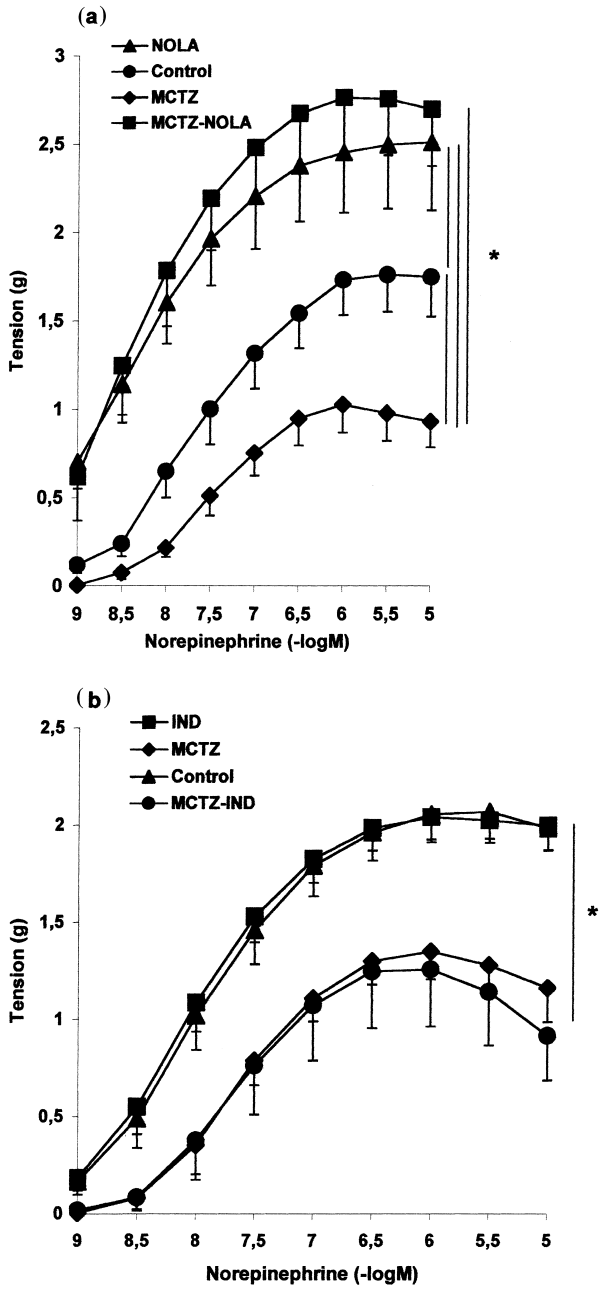


Fig. 2. Effects of *N*ω-nitro-L-arginine (NOLA, $10^{-4}$ M) (a) and indomethacin (IND, $10^{-7}$ M) (b) on methyclothiazide (MCTZ, $10^{-4}$ M) induced inhibition of the contractile response to norepinephrine ($10^{-9}$–$10^{-5}$ M) in SHR aortic rings with functional endothelium ($n = 6$ for each group) ($^{*}P < 0.01$).

### 2.3. Drugs used

MCTZ was a gift from Logeais Laboratories (Paris, France). All other drugs were from Sigma. MCTZ was dissolved in dimethyl sulfoxide (DMSO: final concentration 0.1%). At this concentration, DMSO did not alter the contractions induced by either norepinephrine or $Ca^{2+}$. All other agents were dissolved in distilled water. The drug concentrations are expressed as the final molar concentrations in the bath solution.

MCTZ was prepared according to a method elaborated by Logeais Laboratories. MCTZ was first dissolved as a stock solution in DMSO at a concentration of $2 \times 10^{-1}$ M. This stock solution was then diluted 1:1000 in distilled water to reach the maximal solubility of the compound ($\approx 2 \times 10^{-4}$ M) in water. This latter solution was finally rediluted 1:1 with 2× Krebs–Henseleit medium, giving a final MCTZ concentration of $10^{-4}$ M with 0.1% DMSO. Under these conditions, the actual soluble fraction of MCTZ is around 1% as estimated by UV-spectrographic analysis of the precipitate. Therefore, a solution containing MCTZ at a concentration of $10^{-4}$ M presents an actual concentration of $10^{-6}$ M.

### 2.4. Statistical analysis

In each experimental group, *n* refers to the number of animals from which aortas were taken. Results are shown as means ± S.E.M. Statistical comparisons were performed by means of analysis of variance for repeated measurements and Fisher post-hoc test. Differences were considered to be statistically significant when *P* was less than 0.05.

## 3. Results

In this study, MCTZ did not induce any significant inhibitory effect on the contractile response to norepinephrine in WKY aortic rings, at any concentration. The maximal contractile response induced by norepinephrine was

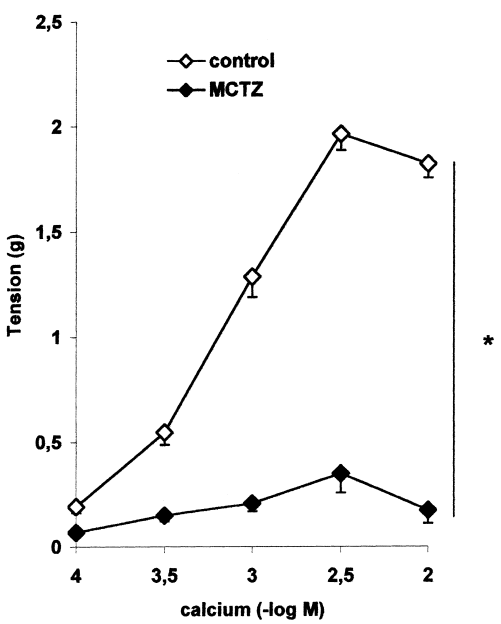


Fig. 3. Inhibitory effect of methyclothiazide (MCTZ, $10^{-4}$ M) on contractions induced by addition of $CaCl_2$ ($10^{-4}$–$10^{-2}$ M) to $Ca^{2+}$-free, high-$K^{+}$ (80 mM) medium, in SHR aortic rings with endothelium ($n = 12$ for each group) ($^{*}P < 0.01$).

inhibited by 16.9 ± 11% ($n = 6$) and 2.3 ± 2% ($n = 6$) in the presence and absence of endothelium, respectively, when MCTZ was perfused at a concentration of $10^{-4}$ M. MCTZ failed to inhibit the contractile responses of SHR rat aorta up to a concentration of $10^{-4}$ M. In the group of endothelium-intact aortic rings, $10^{-4}$ M MCTZ inhibited the contractile response induced by norepinephrine ($10^{-9}$–$10^{-5}$ M). The maximal response to norepinephrine was inhibited by 57.7 ± 7% of the control response (Fig. 1a). MCTZ did not induce any significant relaxation when the endothelium had been removed (Fig. 1b).

The inhibitory effect of MCTZ on the norepinephrine-induced contractile response in endothelium-intact aortic rings was investigated with regards to NOLA or indomethacin treatment, as illustrated in Fig. 2. The basal vascular tone was not significantly affected by any of these treatments, while dose-dependent norepinephrine-induced contraction was only increased by NOLA, reflecting its inhibitory effect on basal NO release. Fig. 2 shows that NOLA (Fig. 2a), but not indomethacin (Fig. 2b) totally abolished the inhibitory effect of MCTZ on the contractile response to norepinephrine.

Interestingly, MCTZ affects the vascular responses to extracellular $Ca^{2+}$ under high-$K^+$ depolarizing conditions. After 30 min in a high-$K^+$, $Ca^{2+}$-free solution, readmission of $Ca^{2+}$ induced a sustained contracture whose amplitude was dependent on the external $Ca^{2+}$ concentration. Fig. 3 shows that in aortic rings with an intact endothelium, MCTZ reduced $Ca^{2+}$ contractures. The maximal contracture was reduced by 90.4 ± 3%.

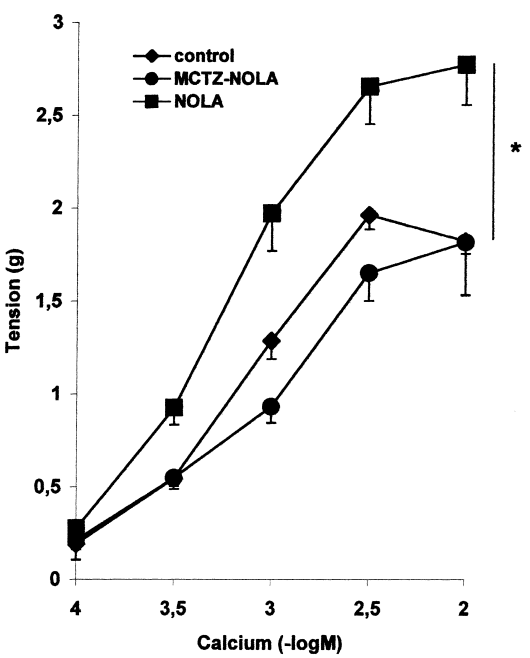


Fig. 4. Effect of *N*ω-nitro-L-arginine (NOLA, $10^{-4}$ M) on methyclothiazide (MCTZ, $10^{-4}$ M) induced inhibition of the contractile responses to $CaCl_2$ ($10^{-4}$–$10^{-2}$ M) in SHR aortic rings with functional endothelium ($n = 6$ for each group) ($^*P < 0.01$).

Pretreatment of endothelium-intact rat aortic rings with NOLA significantly enhanced the contractile response to cumulative addition of $Ca^{2+}$, indicating the inhibitory effect of NOLA on basal and/or voltage dependent NO release. The inhibitory effect of MCTZ on $Ca^{2+}$ contracture was significantly but not totally abolished by the NO synthase inhibitor (Fig. 4).

## 4. Discussion

MCTZ, a thiazide diuretic, induces inhibition of the norepinephrine-induced contractile response of intact vascular rings from SHR, but is ineffective on rings from which endothelium had been removed. These results strongly suggest that MCTZ effects are mediated by the endothelium. It has been demonstrated that endothelium can produce and release two powerful vasorelaxant compounds, EDRF/NO (Furchgott, 1983) and prostacyclin (Moncada, 1980), that may contribute to the endothelium-dependent effect of MCTZ. We therefore decided to investigate the role of both cyclooxygenase and NO synthase pathways on MCTZ-induced relaxation. Our results show that the MCTZ-induced inhibition of the contractile response to norepinephrine is totally abolished by NOLA, an NO synthesis inhibitor, but not by the cyclooxygenase inhibitor, indomethacin. Together, these data demonstrate that the endothelium-dependent effect of MCTZ is mediated by EDRF/NO. The more pronounced effect of MCTZ on arteries from SHR than on those from WKY suggests that NO release could be more important or more efficient in hypertensive animals. This may be due to a compensatory mechanism that hypertensive rat arteries could develop, which could offset the diminished relaxant and/or enhance contractile responsiveness of vascular smooth muscle cells.

It is well known that the reduction of blood pressure by thiazide diuretics is due to reduction of plasma and extracellular fluid volume resulting from the natriuretic and diuretic effects (Villarreal et al., 1988; Greger, 1988). Furthermore, diuretics have been shown to increase both plasma and urinary catecholamines by a reflex-type mechanism such as sympathetic hyperactivity in response to excessive salt and water loss. This effect would blunt the antihypertensive activity of these drugs (Lake et al., 1979; Schiffl et al., 1981). Interestingly, long-term treatment with thiazides shows that they mostly act by lowering peripheral resistance rather than via their diuretic function (Van Brummelen et al., 1980). In this respect, our data suggest that the blood pressure lowering activity of MCTZ is supported by a reduction of the vascular response to endogenous vasoconstricting stimuli such as norepinephrine, via an EDRF/NO-mediated endothelium-dependent mechanism.

It has been reported that the relaxing effect of thiazide diuretics is associated with a fall in $[Ca^{2+}]_i$, which may

explain their direct vasorelaxant effect (Pickkers and Hughes, 1995; Del Rio et al., 1993). Furthermore, some experimental results suggest that this effect on $[Ca^{2+}]_i$ could be mediated by a voltage-dependent $Ca^{2+}$ influx inhibition (Del Rio et al., 1993).

Interestingly, MCTZ induced an inhibition of the contractile response to varying extracellular $Ca^{2+}$ concentration. Moreover, pretreatment with NOLA did not completely abolish this inhibitory effect. The use of this procedure allows the speculation that MCTZ may antagonize voltage-dependent $Ca^{2+}$ channel (VDCC) activity as hypothesized for indapamide (Mironneau, 1988). In that respect, the $Ca^{2+}$ channels would have to be located in the endothelial cells. However, it is controversial whether endothelial cells possess VDCC (for review, see Nilius, 1998). Finally, the VDCC in the vascular smooth muscle cells should also be affected by MCTZ. Together, these arguments tend to eliminate any possible involvement of MCTZ as a $Ca^{2+}$ channel antagonist. The more prominent inhibitory effect of MCTZ observed in high-$K^+$-depolarized aortic rings could be explained by the fact that $K^+$ depolarization of endothelial cells potentiates the release of NO produced through MCTZ treatment. More precisely, Busse et al. (1991) had demonstrated that the endothelial membrane potential plays an important role in endothelial EDRF/NO synthesis. In this context, it seems likely that a $K^+$-induced depolarization could potentiate the release of NO especially when this latter is initially brought up by MCTZ.

Human therapeutic concentrations of MCTZ are usually about $10^{-7}$ M. The need for the elevated concentrations used in the present study can be attributed to the partial water solubility of MCTZ, the short duration of drug application (15 min vs. many weeks in therapeutic use), and the absence of protein binding (Abrahams et al., 1998). The concentrations used in the experiments described here should therefore be considered to be pharmacological doses allowing evaluation of the in vitro effects of MCTZ.

In summary and conclusion, we have shown that MCTZ is responsible for a reduction of the vascular response to the action of endogenous vasoconstricting stimuli such as norepinephrine receptor occupation. According to our data, MCTZ may exert its action by an endothelium/NO-dependent mechanism that could be further reinforced under depolarizing conditions.

## Acknowledgements

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