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Interaction of dimercaptosuccinic acid (DMSA) with angiotensin II on calcium mobilization in vascular smooth muscle cells

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Abstract

Dimercaptosuccinic acid (DMSA) was shown to lower blood pressure in rat models of arterial hypertension. Thus, there is evidence that—besides its chelating properties—DMSA has a direct vascular effect, e.g. through scavenging of reactive oxygen species (ROS). We speculated that, in addition, intracellular calcium mobilization may be involved in this action. Therefore, the present study examined the effects of DMSA on Ca^{2+} mobilization in cultured vascular smooth muscle cells (VSMCs) from rat aorta. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with fura-2 AM. In a first series of experiments DMSA, 10^{-11} to 10^{-6} M, induced an immediate dose-dependent up to 4-fold rise of $[Ca^{2+}]_i$ ($P < 0.001$) which was almost completely blunted by the calcium channel blocker verapamil or the intracellular calcium release blocker TMB-8. In a second series of experiments, when VSMCs were exposed acutely to DMSA (10^{-11} to 10^{-6} M), the angiotensin (ANG) II (10^{-8} M)-induced rise in $[Ca^{2+}]_i$ to 295 ± 40 nM was attenuated at the average by 49% independent of the dose of DMSA. Preincubation of VSMCs with DMSA (10^{-6} M) for 60 min reduced basal $[Ca^{2+}]_i$ by 77% ($P < 0.001$) and dose-dependently attenuated the ANG II (10^{-8} M)-induced rise in $[Ca^{2+}]_i$ between 28 and 69% at concentrations between 10^{-9} and 10^{-5} M DMSA, respectively ($P < 0.05$ and <0.01). In the presence of TMB-8, which attenuated the ANG II (10^{-8} M)-induced rise in $[Ca^{2+}]_i$ by 66%, DMSA (10^{-6} M) had no additional suppressive effect on $[Ca^{2+}]_i$. The results suggest that DMSA acutely raises $[Ca^{2+}]_i$ by stimulating transmembrane calcium influx *via* L-type calcium channels and by calcium release from intracellular stores followed by a decrease in $[Ca^{2+}]_i$ probably due to cellular calcium depletion. Thus, in addition to its action as scavenger of ROS, which in part mediate the vasoconstrictor response, e.g. to ANG II, DMSA may exert its hypotensive effect through decreasing total cell calcium, thereby attenuating the vasoconstrictor-induced rise in $[Ca^{2+}]_i$ in VSMCs.

Keywords: Dimercaptosuccinic acid; Angiotensin II; Calcium mobilization; Rat vascular smooth muscle cell

1. Introduction

Arterial hypertension may result from chronic low level heavy metal intoxication [1,2]. Thus, in a rat model arterial hypertension develops with chronic low level (100 ppm) lead exposure in the absence of morphologic changes in the kidney [3–5]. Associated with the hypertension was a reduced urinary excretion of cGMP as a result of suppressed guanylyl cyclase, the target for NO. Subsequent studies showed that low lead exposure acts by increasing the level of ROS [5].

DMSA, which is a weakly acidic heavy metal chelator to treat lead intoxication [6–9], was shown to prevent or to attenuate hypertension in rats chronically exposed to lead [3,5,10] and to lower blood pressure not only in hypertensive rats with chronic lead intoxication but also in Dahl salt-sensitive and in normotensive rats [11]. These observations suggested that DMSA, acting as a scavenger of ROS because of its sulfhydryl groups, has a direct vascular effect independent of its detoxicating property [10,11]. In the context of more recent evidence which suggests that

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Abbreviations: ANG, angiotensin; $[Ca^{2+}]_i$, intracellular free calcium concentration; cGMP, cyclic guanosine monophosphate; DMSA, dimercaptosuccinic acid; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

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ANG II-induced vasoconstriction is mediated in part by increased production of ROS [12,13] but also by its effect on $[Ca^{2+}]_i$ [14,15], which plays an important role in VSMC contraction, the question arose if DMSA may directly act on $[Ca^{2+}]_i$ and/or may interact with vasoactive hormones such as ANG II by interfering with vasopressor-induced cellular calcium mobilization.

The present study was therefore undertaken to investigate, first, a direct effect of DMSA on $[Ca^{2+}]_i$ and, second, to study its interaction with ANG II on intracellular calcium mobilization in rat VSMC in culture.

2. Material and methods

Studies were performed on VSMCs from the thoracic aorta of male Sprague–Dawley rats weighing 220–260 g which had free access to regular rat chow diet and water.

2.1. Isolation and culture of VSMCs

Rat aortic VSMCs were isolated using a modified method originally described by Chamley *et al.* [16]. Briefly, thoracic aortas from male Sprague–Dawley rats were dissected and incubated in Eagle's minimum essential medium (MEM) containing 2 ng/mL collagenase. After dissecting the adventitia, aortas were minced with sterile razor blades and incubated again for 2–3 hr in MEM containing collagenase. The resulting single-cell suspension was washed and the cells were placed onto 35 mm culture dishes. MEM supplemented with 10% fetal calf serum (FCS) and antibiotics was used as culture medium. The cultures reached confluence after 7–10 days and were then subcultured. Experiments were performed in cells from 3rd to 10th subculture.

2.2. Measurement of intracellular free calcium ($[Ca^{2+}]_i$)

Measurements of $[Ca^{2+}]_i$ were performed by fluorescense photometry according to Hassid [17] with slight modification as previously described [18]. VSMCs were grown on round glass cover slips (13 mm diameter). Upon confluence, the cells were washed and loaded with 4 μM fura-2 AM for 30 min in MEM. The cover slips were then washed three times and rinsed again with buffer containing 2 mM calcium directly before the measurements. For measurements, cover slips were placed into disposable fluorescence cuvettes. Fluorescence measurements were carried out with a Hitachi F4000 fluorescence spectrophotometer which was set to shuttle between the excitation wavelengths of 340 and 380 nm every 4 s. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* [19]. Since baseline $[Ca^{2+}]_i$ differed between VSMCs of different cultures, changes in $[Ca^{2+}]_i$ were also expressed as percent of baseline (100%) values when effects of ANG II or other compounds were compared.

To study the roles of calcium influx *via* L-type calcium channels and of calcium release from the sarcoplasmatic reticulum VSMCs were treated with either the calcium channel blocker verapamil (2×10^{-5} M) or the intracellular calcium release blocker TMB-8 (3×10^{-5} M), respectively, at doses in a range usually employed in *in vitro* studies on rat VSMCs [20,21] and in our laboratory [15]. They were added 10 min before starting measurements of $[Ca^{2+}]_i$.

Fura-2 AM (fluorescence indicator), TMB-8 (3,4,5-trimethoxy-benzoic acid 8-[diethylamino] octyl ester), verapamil and angiotensin II were purchased from Sigma. DMSA was provided by McNeil.

2.3. Statistical analysis

For statistical analysis ANOVA and Student's *t*-test were employed. Data are presented as means ± SEM.

3. Results

3.1. Effects of DMSA on $[Ca^{2+}]_i$ in VSMCs

In a first series of experiments cultured VSMCs revealed an average baseline $[Ca^{2+}]_i$ of 21 ± 3 nM. DMSA, when added to VSMCs in the culture medium, resulted in a rapid rise in $[Ca^{2+}]_i$ to peak concentration which was followed by a slow return towards baseline over the following 8–10 min. At concentrations ranging from 10^{-11} M (which had no significant effect on $[Ca^{2+}]_i$) to 10^{-6} M DMSA increased $[Ca^{2+}]_i$ dose-dependently from 100 ± 14% (baseline) to 381 ± 38%, i.e. 80 ± 9 nM ($P < 0.001$) (Fig. 1, lower panel; Fig. 2).

Verapamil (5×10^{-5} M), when added to the culture medium for 10 min, reduced basal $[Ca^{2+}]_i$ to 16 ± 2 nM ($P < 0.001$) and suppressed the immediate DMSA (10^{-6} M)-induced rise in $[Ca^{2+}]_i$ from 80 ± 9 to 24 ± 4 nM ($P < 0.01$) (Fig. 2). Similarly, TMB-8 (3×10^{-5} M), when added to VSMCs in culture for 10 min, reduced basal $[Ca^{2+}]_i$ to 14 ± 2 nM ($P < 0.001$) and suppressed the initial rise in $[Ca^{2+}]_i$ induced by DMSA (10^{-6} M) to 25 ± 4 nM ($P < 0.01$) (Fig. 2).

3.2. Interaction of DMSA with ANG II

In a second series of experiments VSMCs in culture revealed an average $[Ca^{2+}]_i$ of 48 ± 3 nM. ANG II at concentrations between 10^{-11} and 10^{-6} M induced a rapid dose-dependent rise in $[Ca^{2+}]_i$ from 100 ± 6% (baseline) to 631 ± 52%, i.e. 303 ± 25 nM ($P < 0.001$) (Fig. 1, upper panel).

ANG II at a concentration of 10^{-8} M increased $[Ca^{2+}]_i$ to 295 ± 40 nM ($P < 0.001$). This rise in $[Ca^{2+}]_i$ was reduced in the presence of verapamil (5×10^{-5} M) by 56% to 156 ± 26 nM ($P < 0.01$) and in the presence of TMB-8 (3×10^{-5} M) by 66% to 131 ± 9 nM ($P < 0.005$)

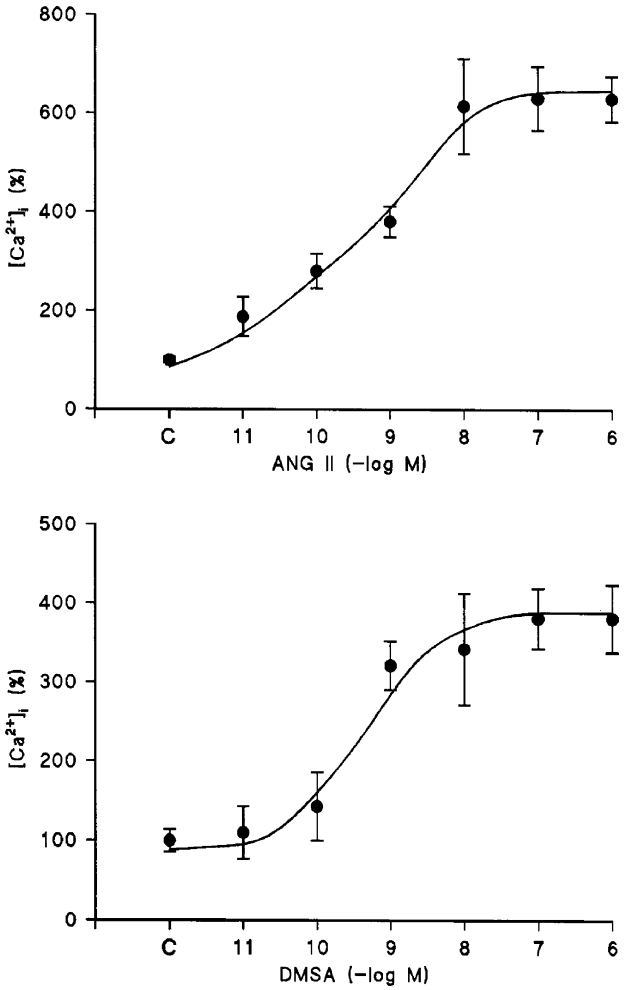

Fig. 1. Dose–response curves of the rise in $[Ca^{2+}]_i$ in cultured rat VSMCs immediately after addition of DMSA (bottom) and of angiotensin II (ANG II) (top) at concentrations ranging from 10^{-11} to 10^{-6} M, respectively. Each point represents mean ± SEM of N = 6–12 experiments. Note the different scales of *y*-axis in top and bottom figures.

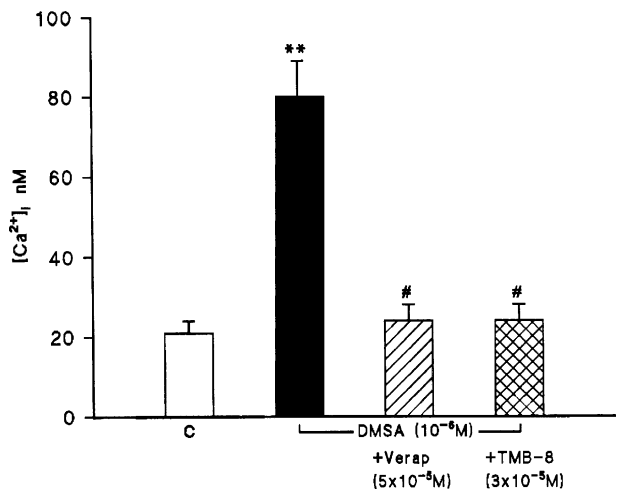

Fig. 2. Effects of verapamil and TMB-8 on the immediate response of $[Ca^{2+}]_i$ in rat VSMCs to the addition of DMSA, 10^{-6} M. Each bar represents mean ± SEM of N = 12 experiments; $^{**}P < 0.001$ vs. control (C); $^{\#}P < 0.01$ vs. DMSA.

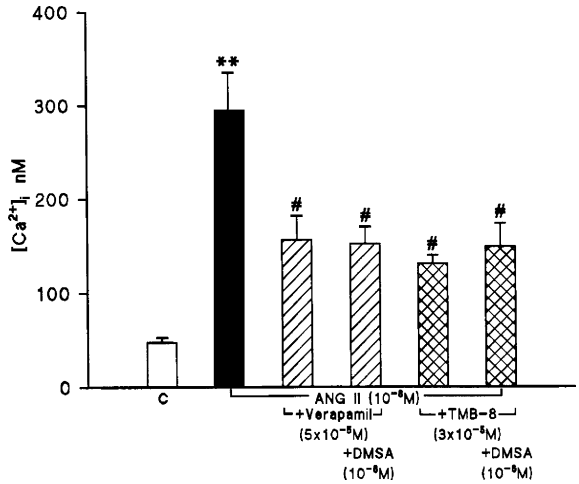

Fig. 3. Effects of 10 min preincubation of rat VSMCs with verapamil or TMB-8 on the angiotensin II (ANG II; 10^{-8} M)-induced rise in $[Ca^{2+}]_i$. No effect of DMSA (10^{-6} M) in addition to that of verapamil or TMB-8 was observed when DMSA was added 2 min before the addition of ANG II. Each bar represents mean ± SEM of each N = 6 experiments. $^{**}P < 0.01$ vs. control (C); $^{\#}P < 0.01$ vs. ANG II.

(Fig. 3). When ANG II (10^{-8} M) was added to the incubation medium 2 min after the addition of DMSA at concentrations ranging from 10^{-11} to 10^{-6} M, DMSA suppressed the ANG II-induced rise in $[Ca^{2+}]_i$ independent of the dose of DMSA at the average by 45% to 184 ± 24 nM ($P < 0.01$).

The suppression of the ANG II (10^{-8} M)-induced rise in $[Ca^{2+}]_i$ by 10 min preincubation of VMSCs with verapamil or TMB-8 was not affected by DMSA (10^{-6} M), when DMSA was added to the incubation medium 2 min prior to the addition of ANG II. $[Ca^{2+}]_i$ in the presence of ANG II and DMSA in verapamil- and TMB-8-pretreated VSMCs was 152 ± 18 and 149 ± 25 nM, respectively (Fig. 3).

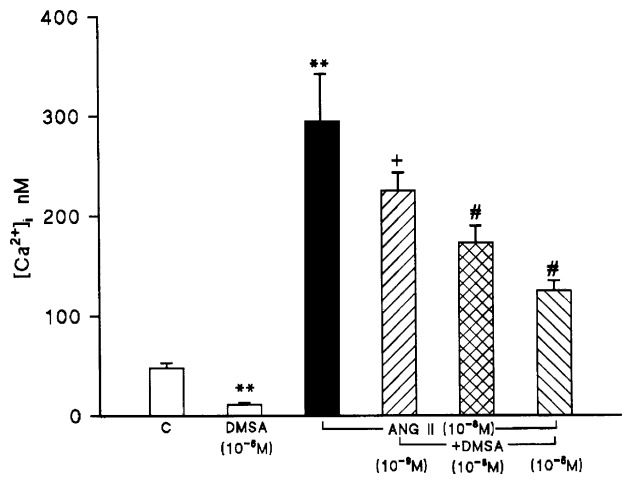

Fig. 4. Effects of 60 min preincubation with DMSA (10^{-6} M) on basal $[Ca^{2+}]_i$ (2nd bar from left vs. C) and effects of DMSA at concentrations ranging from 10^{-9} to 10^{-5} M on the angiotensin II (ANG II; 10^{-8} M)-induced rise in $[Ca^{2+}]_i$ (solid bar) in rat VSMCs. Each bar represents mean ± SEM of each N = 7 experiments; $^{**}P < 0.001$ vs. C; $^{+}P < 0.05$ and $^{\#}P < 0.01$ vs. ANG II.

DMSA added for 60 min to the culture medium at concentrations of 10^{-9} and 10^{-6} M significantly reduced basal $[Ca^{2+}]_i$ of VSMCs to 23 ± 3 ($P < 0.01$) and 11 ± 2 nM ($P < 0.001$; Fig. 4), respectively.

When VSMCs were incubated with DMSA for 60 min at concentrations of 10^{-9}, 10^{-6} and 10^{-5} M, DMSA dose-dependently attenuated the ANG II (10^{-8} M)-induced rise in $[Ca^{2+}]_i$ by 28% to 225 ± 18 nM, by 49% to 173 ± 17 nM and by 69% to 125 ± 10 nM, respectively (each N = 7; $P < 0.05$ and <0.01, respectively (Fig. 4).

4. Discussion

DMSA belongs to a new generation of chelators and has been used since 1989 as a first-line drug in lead intoxication [7]. In experimental lead nephropathy DMSA was shown to restore glomerular filtration rate and to reduce albuminuria [3] despite the persistence of morphologic changes. The same authors showed in this animal model a significant antihypertensive action of DMSA. Animals which had developed hypertension with administration of lead (0.01% in drinking water) for 6 months were treated during the subsequent 6 months at three time periods each of 5 days with DMSA (0.5% in drinking water) which resulted in a significantly lower blood pressure than in untreated rats [11]. This effect could not be attributed to DMSA as a chelator since blood lead concentration was similar at the end of the 12 months in rats given lead alone or lead plus subsequent DMSA treatment. In subsequent studies in Dahl salt-sensitive rats the observed antihypertensive effect of DMSA was conjectured to be related rather to direct hemodynamic vascular changes [10]. Since $[Ca^{2+}]_i$ in VSMCs plays an important role in vascular resistance [18] and thus in the pathogenesis of arterial hypertension, it was of interest to investigate the effects of DMSA on $[Ca^{2+}]_i$ in VSMCs in culture and its interaction with the potent vasoconstrictor ANG II.

DMSA, when applied directly to VSMCs at concentrations between 10^{-11} and 10^{-6} M, resulted in a dose-dependent immediate rise in $[Ca^{2+}]_i$ which was much smaller than that induced by ANG II. In contrast to the immediate rise in $[Ca^{2+}]_i$ induced by ANG II, which rapidly returns to control values as we [14] and others [22] showed previously and as was found in the present study, the DMSA-induced rise in $[Ca^{2+}]_i$ only slowly returned toward baseline. It is noteworthy that the maximum concentration of DMSA (1 μM) used in these experiments is far below the peak plasma concentration of 20 μM reached in patients treated with DMSA [23].

Both, verapamil, a L-type calcium channel blocker, which due to its lipophilic property may also enhance NOS similar to what was shown for other calcium channel blockers (see [24]), and TMB-8, a blocker of calcium release from the sarcoplasmatic reticulum, inhibited the acute calcium mobilizing effect of DMSA similar to their effects on the ANG II-induced rise in $[Ca^{2+}]_i$. This observation suggests that increased transmembranous calcium influx and enhanced release of calcium from the intracellular stores contribute to the initial rise in $[Ca^{2+}]_i$ after exposure of VSMCs to DMSA. Moreover, these data make it unlikely that the effects of DMSA could have resulted from DMSA's metal chelating property, i.e. binding of intracellular free calcium ions.

As an immediate interaction of DMSA with ANG II we found an attenuation of the ANG II-induced rise in $[Ca^{2+}]_i$ which was independent of the dose of DMSA. Exposure of VSMCs to DMSA for 60 min resulted in a significant decrease in basal $[Ca^{2+}]_i$ and in a significant dose-dependent suppression of the ANG II-induced rise in $[Ca^{2+}]_i$. DMSA, however, had no additional effect on the attenuation of the ANG II-induced rise in $[Ca^{2+}]_i$ by verapamil or TMB-8. In these *in vitro* studies an action of a DMSA–cysteine mixed disulfide, that is described *in vivo* as an excretion product of DMSA (where cysteine apparently originates from the plasma) [25], is unlikely but cannot be ruled out completely.

The initial rise in $[Ca^{2+}]_i$ in VSMCs which we observed with DMSA is not unusual for vasoactive agents even though they are potent vasodilators. Thus, we have found previously that bradykinin, a potent vasodilator peptide, produced an initial effect with a transient rise in $[Ca^{2+}]_i$ (blocked by the B_2-receptor antagonist HOE 140 and attenuated in calcium-free medium or by TMB-8) and a prolonged effect with attenuation of the ANG II-induced rise of $[Ca^{2+}]_i$ in VSMCs [15]. Also similar to the effects of DMSA we found direct effects of the ACE-inhibitor ramiprilat on $[Ca^{2+}]_i$ in VSMCs [26], consisting of an immediate rise in basal $[Ca^{2+}]_i$, which was attenuated by TMB-8, and of a delayed effect consisting of marked attenuation of calcium mobilization by ANG II, most probably due to depletion of intracellular calcium stores. While exerting similar effects on calcium kinetics in VSMCs as does DMSA, bradykinin and ramiprilat may act, in addition, through increased iNOS-derived NO production ([27], Meyer-Lehnert *et al.*, unpublished data), whereas DMSA scavenges ROS probably by its thiol groups [11]. Both these mechanisms will provide greater availability of NO to modulate intracellular calcium metabolism.

Thus, Khalil-Manesh and co-workers [10] hypothesized that the antihypertensive effect of DMSA may at least in part be mediated through the second messenger cGMP by virtue of scavenging ROS and thus increasing NO [28]. As mentioned above, rats, which were given lead and had developed hypertension, revealed increased generation of malondialdehyde, a measure of lipoperoxide production [3,28]. Animals with the same lead exposition but treated with DMSA were found to have elevated plasma concentrations of cGMP [11]. It was shown that cGMP *via* protein kinases inhibits transmembranous calcium influx [29] and also inhibits calcium mobilization from intracellular stores in contractile cells

[30] possibly through inhibition of phospholipase C or protein kinase C or receptor- and voltage-dependent calcium channels [31]. cGMP also may influence IP_3-production and thereby affect $[Ca^{2+}]_i$ [32]. Thus, DMSA may modulate intracellular calcium metabolism either directly *via* its effects on cellular calcium entry and release or indirectly as ROS scavenger *via* enhanced NO and cGMP formation.

In summary, on one hand, DMSA as antioxidant [11] was shown *in vitro* and *in vivo* to scavenge ROS and to increase NO formation [11,28]. On the other hand, DMSA has an immediate effect on $[Ca^{2+}]_i$ of VSMCs and, when acting in a prolonged manner, it reduces basal $[Ca^{2+}]_i$, which is also essential for enzymes involved in the generation of ROS [21]. DMSA blunts the responses of $[Ca^{2+}]_i$ to vasoconstrictors such as ANG II, as shown in the present study, and it may suppress the rise in $[Ca^{2+}]_i$ in response to oxygen-derived free radicals [33,34]. These mechanisms are likely to underlie the vasodilatory action of DMSA and may become increasingly attractive, since there is accumulating evidence for complementary roles of intracellular calcium and of ROS production in the pathogenesis of ANG II-induced and other forms of arterial hypertension [12,13].

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