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## EFFECTS OF GADOLINIUM CHLORIDE ON BASAL FLOW AND COMPRESSION-INDUCED RAPID HYPEREMIA IN THE RABBIT MASSETER MUSCLE

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Aim of the present study is to investigate the role of mechano-sensitive channels on basal muscle blood flow and on the compression-induced rapid hyperaemia. To this aim, the mechano-sensitive channel blocker Gadolinium ( $Gd^{3+}$ ) is employed, which already proved to reduce the myogenic response in isolated vessels. Muscle blood flow (MaBF) was recorded from the masseteric artery in 8 urethane-anesthetized rabbits. Rapid hyperemic responses were evoked by 1-s lasting compressions of the masseter muscle (MC) delivered before and after close arterial infusion of  $Gd^{3+}$  in the masseteric artery. Three infusions were performed at 1-h interval, producing estimated plasma concentration (EPC) of 0.045, 0.45 and 4.5 mM, in the masseteric artery. The amplitude of the hyperaemic response to MC, equal to  $195\pm 77\%$  of basal flow in control condition, was reduced by  $9.5\pm 19.4\%$  ( $p=0.18$ ) and  $45\pm 28\%$  ( $p<0.01$ ) while basal MaBF increased by  $10\pm 3\%$  ( $p=0.90$ ) and by  $68\pm 30\%$  ( $p<0.01$ ) at EPC of 0.045 and 0.45 mM, respectively. At EPC of 4.5 mM a strong reduction in both MaBF (by  $54\pm 13\%$ ,  $p<0.01$ ) and MC response ( $75\pm 12\%$ ,  $p<0.01$ ) was instead observed. These effects did not depend on time from infusion. At all doses employed  $Gd^{3+}$  never affected arterial blood pressure, heart rate and contralateral MaBF. While the effects observed at the highest EPC likely result from blood vessel occlusion due to  $Gd^{3+}$  precipitation, the effects observed at lower concentrations demonstrate that  $Gd^{3+}$  affects musculo-vascular function by decreasing both resting vascular tone and responsiveness to mechanical stimuli. The results are compatible with a  $Gd^{3+}$ -induced blockade of vascular mechano-sensitive channels.

Key words: *gadolinium, muscle blood flow, mechano-transduction, rapid dilatation, mechano-sensitive channel, myogenic effect, masseter muscle*

### INTRODUCTION

Several studies have recently focused on the rapid vasodilation elicited by external muscle compression (1-3) suggesting that, like the classical myogenic response, it is at least partly mediated by mechanosensitive mechanisms responding to a reduction in transmural pressure (2, 4, 5).

Understanding the mechanisms of vascular mechano-sensitivity is of primary importance for its potential contribution to the regulation of vascular tone and, consequently, of vascular resistance and arterial blood pressure (6-8).

Mechano-sensitivity of blood vessels is considered to rely mostly on mechano-sensitive channels (MSC), largely expressed by both endothelium and vascular smooth muscle (2, 9). Selective blockade of these channels has been attempted in *in vitro* studies (10, 11) by means of different substances such as 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), tarantula protein (GsMtx2), gadolinium ( $Gd^{3+}$ ) (10). Although being characterized by low specificity (12), gadolinium ( $Gd^{3+}$ , a trivalent lanthanide), has been widely used as MSC blocker, to test mechano-sensitivity of single cells (11) and isolated vessel preparations (13). In particular  $Gd^{3+}$  was shown to provoke

marked attenuation of the classical myogenic response (13), *i.e.* the slow (lasting about 1 min) vascular constriction/dilatation occurring in response to step increase/decrease in the vessel's transmural pressure. However, to our knowledge the effect of MSC blockade has never been tested on the rapid dilatatory response induced by a short lasting muscle compression.

Aim of the present work is to test the role of mechano-sensitive ion channels on the rapid hyperaemic response induced by external compression of the masseter muscle in anesthetized rabbits, by assessing the effect of  $Gd^{3+}$  administration. A recently developed model has been employed, which allows for accurate monitoring of blood flow from the purely muscular masseteric artery (14). This vascular bed appears to be very reactive to mechanical stimuli and the ensuing hyperaemic events can be produced with high repeatability by muscle compression (5, 15).

One of the reason why  $Gd^{3+}$  has been little investigated *in vivo* is related to its toxic effect, possibly resulting in hypotension, cardiac arrhythmias, A-V bundle block, ventricular fibrillation when administered systemically (16). In order to overcome acute collateral effects and prevent disturbing cardiovascular changes at systemic level,  $Gd^{3+}$  was here locally administered in small doses through close arterial injection.

## MATERIAL AND METHODS

The study was performed in accordance with the principles of laboratory animal care. Purposes and protocols were approved by the Ethical Committee for Animal Experiments at the University of Turin.

*Surgical procedure*

Experiments were carried out on 8 male European rabbits (*Oryctolagus cuniculus*) weighing between 3.1 and 3.9 kg, anesthetized with urethane (Urethane, Sigma Aldrich, 1.2 g/kg i.v.). Full surgical anaesthesia was maintained by injecting additional doses of the drug (0.4 g/kg i.v.) through a catheter inserted in the cannulated left femoral vein. In all animals trachea was intubated and the head was fixed in a stereotaxic frame by screws implanted in the nasal and frontal bones.

A telemetric pressure transducer (TA11PA-D70, DSI - St. Paul, MN, USA) was implanted, the catheter being inserted into the right femoral artery for monitoring arterial blood pressure (ABP).

A perivascular flow probe (model 0.7 PSB, Transonic Systems Inc, Itaha, NY, USA) was implanted on the masseteric branch of the facial artery (Ma). The Ma, which exclusively supplies the rostral portion of the masseter muscle, was isolated medially to the mandibular margin, immediately after its branching from the facial artery, for placement of the probe (14). In 5 of 8 animals a bilateral implant was performed so that blood flow could be recorded simultaneously from the left and right masseteric artery.

A silicone cannula (diameter 0.5 mm, CNC-3IS-LSA/LSP Access Technologies, USA) was inserted at the distal mandibular end of the facial artery and advanced proximally, up to the origin of the Ma, for close arterial injection of  $Gd^{3+}$  in the masseter muscle. The cannula was continuously perfused with saline solution at 2–3 ml/h (infusion pump Terumo Europe STC-521, Leuven, Belgium) to prevent clotting.

The experimental procedures started after stabilization of the hemodynamic variables, about 1 hour after the surgical preparation was completed. At the end of the experiments animals were killed with an i.v. injection of a lethal dose of urethane.

*Drugs*

Gadolinium chloride ( $Gd^{3+}$ , Sigma, Steinhein, Germany), an antagonist of stretch-activated cation channels, was dissolved in

HEPES buffer (pH 7.3–7.4) (17–19) and infused at increasing concentrations of 0.1 mM, 1.0 mM and 10 mM into the cannulated facial artery, through an infusion pump (Terumo STC-521, Terumo Co., Tokyo). The rate of injection was set to 50% of basal blood flow in the masseteric artery, ranging between 6 and 24 ml/h, for a duration of 2 minutes. This approach was aimed to obtain the same  $Gd^{3+}$  plasma concentration in all masseteric arteries. Given that rabbit haematocrit is about 40% (20), the estimated plasma concentration (EPC) of  $Gd^{3+}$  in masseteric artery during drug infusion approximately corresponds to 0.045–0.45–4.5 mM for the three dosages employed. The three administrations were separated by 1-h lasting intervals, during which changes in basal blood flow and vascular reactivity were assessed.

*Mechanical stimulation*

Mechanical compression of the masseter muscle was performed unilaterally by means of a shaker (model V2, Data Physics Corporation, San Jose, CA USA), driven by a computer-generated trapezoidal signal (rise time: 0.1 s; plateau: 1 s; return: 0.1 s), delivered through a 1401 micro I/O board (CED, Cambridge, UK). The shaker was armed with a cylindrical head (diameter of about 1.3 cm, amplitude of movement up to 5 mm) positioned so that it exerted appropriate and repeatable compressions of the cheek at the level of the anterior portion of the masseter muscle (5). Mechanical stimuli were delivered in series of 10 MCs separated by 30-s intervals. This inter-stimulus interval was maintained precisely constant (PC-controlled) since it was observed to affect the amplitude of the hyperemic response to MC (15).

One series was delivered before the first  $Gd^{3+}$  administration (control), then other series were periodically delivered at 3.5, 15, 30 and 45 min after the end of each  $Gd^{3+}$  infusion, as illustrated in Fig. 1.

*Data acquisition and processing*

Acquisition of all signals and off-line processing were performed with Spike2 (CED, Cambridge, UK). Simple algorithms were implemented in the Spike2 script language aimed at identifying single cardiac cycles (based on systolic peak detection on the ABP signal), from which time averages of the different signals (one value per cardiac cycle) were computed. Measurement of ABP, heart rate (HR) and masseteric

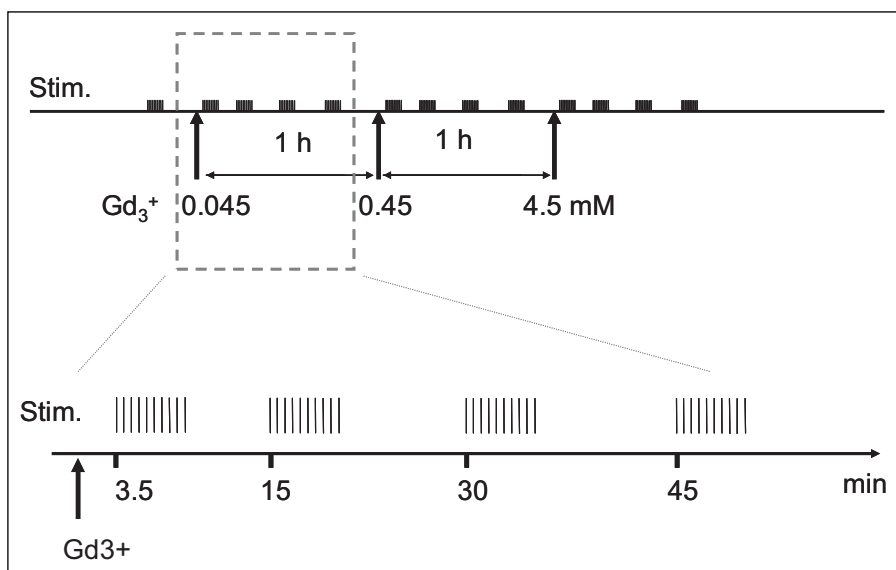


Fig. 1. Scheme describing the experimental protocol mechanical stimulation. It was composed by 5 series of 10 MC (30 second between each one). The series was started before  $Gd^{3+}$  administration (control), after 3.5 minutes ( $t_{3.5}$ ), after 15 minutes ( $t_{15}$ ), after 30 minutes ( $t_{30}$ ) and after 45 minutes ( $t_{45}$ ). This sequence was repeated (0.045 mM–0.45 mM–4.5 mM).

artery blood flow ipsilateral (iMaBF) and contralateral (cMaBF) to the side of mechanical stimulation and of  $Gd^{3+}$  injection were continuously performed. The blood flow response to MC was characterized in terms of peak flow response and peak amplitude (=peak flow-basal flow). These measurements were taken before and 4 times after each  $Gd^{3+}$  injection (at 3.5, 15, 30, 45 min, after injection). Basal flow and peak flow of hyperaemic responses were normalized to the basal flow of the control condition (*i.e.*, before the first  $Gd^{3+}$  injection). In addition, the peak amplitude of the hyperaemic response was normalized to the pre-stimulus level. For all variables the average effects were computed across all animals ( $n=8$ ) and reported as mean  $\pm$  standard deviations (S.D.).

Statistical significance of the effect of  $Gd^{3+}$  on the measured variables was assessed with a two-ways ANOVA and Duncan Post hoc test, with time from injection (time: 3.5, 15, 30, 45 min) and  $Gd^{3+}$  concentration (EPC: 0.045, 0.45, 4.5 mM) as factors.

## RESULTS

In control conditions MaBF ranged between 0.2 and 1 ml/min, on average  $0.44 \pm 0.08$  ml/min. Compression of the masseter muscle produced a transient reduction in iMaBF, followed by a rapid hyperaemic response, developing immediately after release of the stimulus (peak amplitude:  $0.85 \pm 0.33$  ml/min, time to peak:  $2.2 \pm 0.8$  s) and returning to control within 10–15 sec. A representative example of the hyperaemic response to subsequent stimuli is shown in *Fig. 2A*.

Neither ABP nor HR nor cMaBF were statistically dependent on the time from  $Gd^{3+}$  injection ( $p=0.74$ ) or on the

concentration of the drug ( $p=0.95$ ), indicating that  $Gd^{3+}$  did not reach the general circulation at a concentration sufficient to be effective. On average, ABP, HR and cMaBF were  $96.1 \pm 5.8$  mmHg,  $308 \pm 10$  bpm and  $0.36 \pm 0.13$  ml/min, respectively.

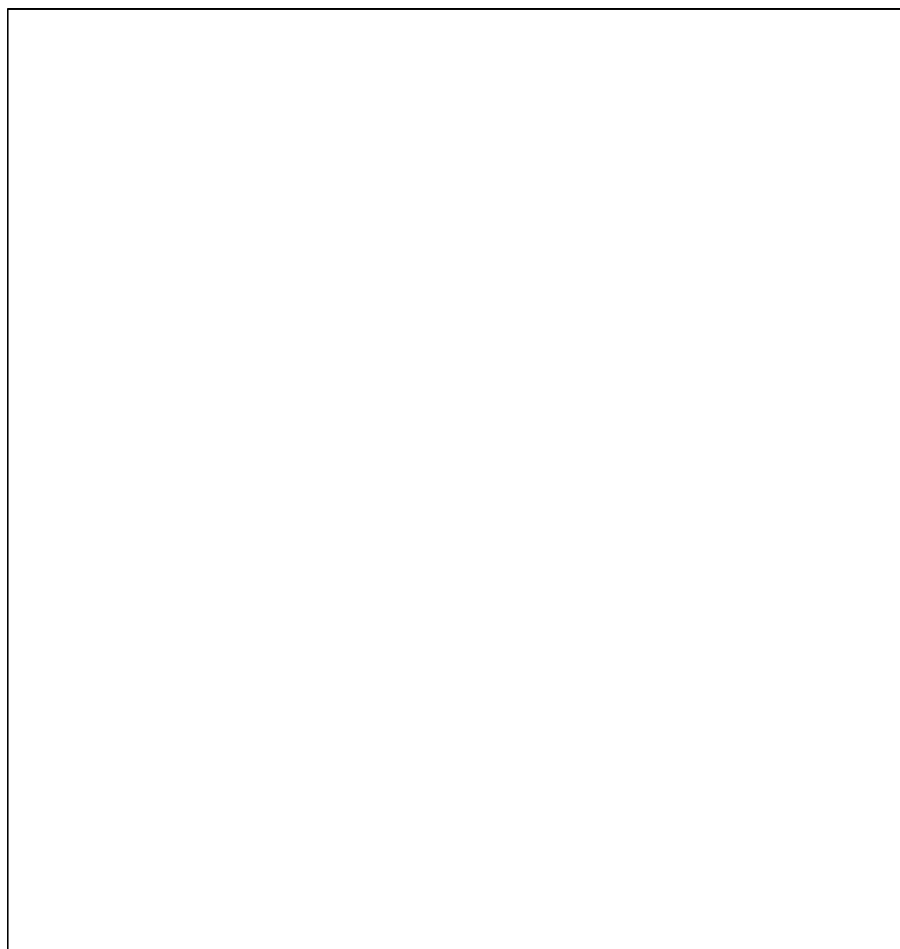
Administration of  $Gd^{3+}$  at increasing concentration progressively decreased the amplitude of the hyperaemic response to MC, while also affecting basal blood flow. A representative example is shown in *Fig. 2B* in which the average responses to MC collected 45 min after  $Gd^{3+}$  administration at different concentrations (progressively lighter grey traces corresponding to increasing concentrations) are superimposed to the MC response in control condition (black trace). It can be observed that the decrease in the amplitude of response occurs independently of an effect on basal blood flow.

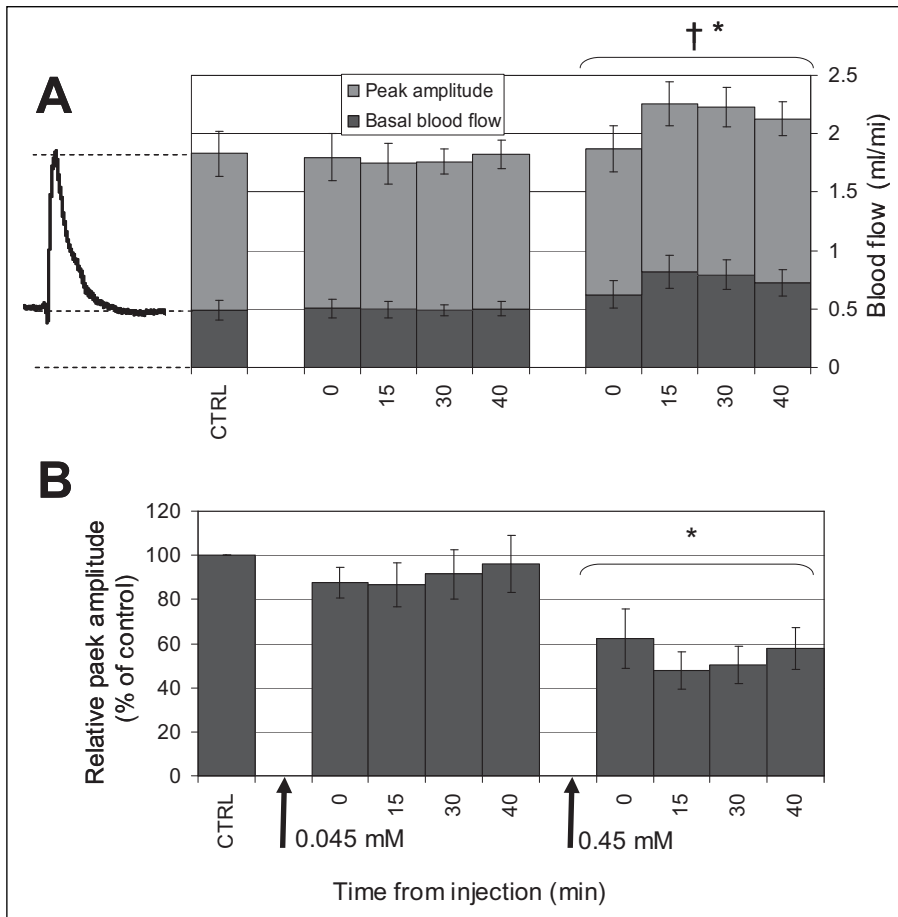
The time course of peak amplitude (light grey) and basal flow (dark grey) is reported in *Fig. 3A*, the full height of each bar thus representing peak flow. None of the variables exhibited a dependence on time from  $Gd^{3+}$  administration (peak flow:  $p=0.51$ ; basal flow:  $p=0.20$ ; peak amplitude:  $p=0.45$ ) but all showed a dependence on concentration ( $p<0.01$ ).

After averaging over time, peak flow at the different EPCs was  $1.2 \pm 0.2$  ml/min at 0.045 mM,  $1.4 \pm 0.2$  ml/min at 0.45 mM and  $0.25 \pm 0.1$  ml/min at 4.5 mM (not shown), only at the latter concentration being significantly different from the control condition ( $1.3 \pm 0.2$ ) ( $p<0.01$ ).

Basal masseter blood flow was  $0.4 \pm 0.08$  ml/min at the control, increased to  $0.5 \pm 0.11$  ml/min at 0.045 mM ( $p=0.9$ ) and  $0.73 \pm 0.18$  ml/min ( $p<0.01$ ) at 0.45 mM and decreased to  $0.22 \pm 0.18$  ml/min at 4.5 mM ( $p<0.01$ ).

The peak amplitude decreased from  $0.85 \pm 0.33$  ml/min during the control to  $0.64 \pm 0.25$  ml/min at 0.45 mM ( $p<0.01$ ) as





**Fig. 3.** Average effects of  $Gd^{3+}$  on basal flow and peak amplitude of the MC response. (A) average basal flow (dark grey) and peak amplitude (light grey) are reported before (CTRL) and at the different time intervals after close arterial injection of  $Gd^{3+}$  leading to estimated plasma concentration of 0.045, and 0.45 mM. As illustrated by the representative MC response reported on the left, the total height of each bar corresponds to average peak flow. (B) Relative peak amplitude, *i.e.*, referred to current basal flow is reported in this bar diagram to highlight the reduction induced by  $Gd^{3+}$ . All values have been normalized to control value (CTRL). The presentation of the bar diagram reflects the experimental protocol, with arrows at the bottom indicating  $Gd^{3+}$  administration and labels indicating the corresponding estimated plasma concentrations (0.045, 0.45 mM). Data corresponding to EPC of 4.5 mM were not reported (see Discussion). † and \* indicate significant difference with respect to the control condition ( $p < 0.01$ ) for basal flow and peak amplitude, respectively. Error bars indicate standard error ( $n=8$ ).

shown in Fig. 3A. However, since basal flow was concomitantly increased, the relative peak amplitude (=peak amplitude / basal flow) was markedly attenuated by gadolinium at EPC=0.45 mM. This effect is represented in Fig. 3B in which the average relative peak amplitude is reported after normalization to control value, taken as 100%. Relative peak amplitude was reduced from  $195 \pm 77\%$  of baseline, in control condition, to  $100 \pm 42\%$  (at 0.45 mM, EPC), corresponding to an average reduction of  $45 \pm 28\%$ .

## DISCUSSION

For the first time the vascular effect of  $Gd^{3+}$  were investigated *in vivo*, by means of our recently-developed experimental model (5).

The results showed that close arterial injection of  $Gd^{3+}$ , resulting in estimated plasma concentrations of 0.045 and 0.45 mM, progressively reduced the amplitude of the rapid hyperaemic response to external muscle compression (to 65% of control) while increasing basal blood flow (to 68% of control). The marked effects observed at the highest concentration (4.5 mM) deserve a more careful consideration being possibly produced by  $Gd^{3+}$  precipitation. Gadolinium administration did not affect systemic variables such as ABP and HR, nor blood flow in the contralateral masseteric artery.

Arterioles and small arteries usually develop and maintain some degree of active force at their normal intravascular pressure, allowing resistance to be modulated in two directions, vasodilatation and vasoconstriction. In his famous model Johnson (21) put forward the idea that arteriolar wall tension, rather than the vessel diameter, is the regulated variable so that vessel diameter is decreased in response to increased transmural

pressure, according to the Laplace's law. This model also explains the "spontaneous" myogenic tone that develops in resting conditions, in relation to intraluminal pressure (22). The mechano-sensitive channels (MSC), largely expressed in both endothelium and smooth muscle of blood vessels, are considered to be involved in the vascular mechano-sensitivity (2, 9) that mediates the myogenic response (9) as well as in the maintenance of basal tone (12, 23-25).

Although a unique blocker of these mechano-sensitive channels has not been identified, gadolinium chloride is considered an effective mechano-sensitive channel blocker (26, 27) and its action on these channels has been documented by a large numbers of *in vitro* studies performed on different tissues, including vascular smooth muscle (26) and endothelium (28, 29).

In the present study, close arterial injection of  $Gd^{3+}$  resulted in increased resting blood flow ( $69 \pm 71\%$  at 0.45 mM), in the absence of changes in arterial blood pressure. This reveals a dilatation of resistance vessels and, to our knowledge, is the first observation *in vivo* of the involvement of MSC channels in resting myogenic tone. This result is in agreement with the hyperpolarizing effect observed in isolated cerebral arteries upon exposure to  $Gd^{3+}$  ( $30 \pm M$ ), which was attributed to a depressant action exerted by the drug on non selective cation channels (30). However, a dilatatory effect has not been consistently observed in *in vitro* studies (11, 23, 25), possibly due to the fact that single different vascular segments were investigated. Such a limitation does not apply to our *in vivo* model in which the whole musculo-vascular tree of the masseter muscle has been exposed to the drug. Interestingly a dilatatory effect of  $Gd^{3+}$  is compatible with the general hypotensive effect observed upon systemic administration by *i.v.* injection in cats (16), although in that study specific vascular effects were not investigated.

The muscle vascular bed is known to have a large dilatory capacity and MaBF was frequently observed to increase above 10 times the basal value in this same preparation, during functional hyperaemia (14). On this basis, the decrease in the hyperaemic response to MC cannot be attributed to a saturation of the dilatory capacity associated to the increase in basal blood flow. Moreover, in some instances the reduction in the MC response was accompanied by negligible effects on basal blood flow, thus resulting in a net reduction of peak flow (*e.g.*, Fig. 2, EPC=0.045 mM). These considerations indicate that the effect on MC response is distinct from the effect on basal flow.

It is known that an increase in transmural pressure activates the mechano-sensitive non-selective cation channels leading to depolarization of vascular smooth muscle cells and increase of intracellular  $[Ca^{++}]$  which underlies the vasoconstriction according to the classical myogenic response (27). Opposite effects are considered to mediate the vasodilatation that follows a reduction in transmural pressure, although this phenomenon has been comparatively less investigated (9). Exposure to  $Gd^{3+}$  was shown to completely abolish the myogenic response in isolated afferent arterioles of the rabbit kidney at a concentration of 10  $\mu M$  (13), while the same concentration was ineffective in the rabbit basilar artery (31), suggesting a large variability among different vascular compartments. In this respect, the musculo-vascular compartment is known to exhibit a prominent and rapid dilatation in response to mechanical stimulation, particularly to stimuli that decrease transmural pressure, *e.g.*, compression of the muscle (1, 3, 4, 32, 33) and occlusion of the supplying artery (1). In addition, this rapid dilatory response has been observed also in isolated muscle feed arteries (2) and arterioles (25) subjected to external compression and to a drop in internal pressure, respectively. In the latter study the authors showed that the peak amplitude of the rapid dilatation following transient reduction of intraluminal pressure (80–10–80 mmHg) was significantly reduced by  $Gd^{3+}$  (10  $\mu M$ ), supporting the idea that MSCs mediate at least part of the response (25). The effect was however observed only for stimulus duration of 60 and 120 s and not for duration of 30 s. The MC stimulus adopted in the present study likely produces a similar decrease in transmural pressure of intramuscular blood vessels but, at difference with the above mentioned study it lasts only 1 s. Moreover the presently observed effects concern the response of the whole musculo-vascular network, not just the response of the arteriolar segment. Thus, although collected at higher theoretical  $Gd^{3+}$  concentration (EPC=450  $\mu M$ , discussed below), the present results extend the observation of Koller & Bagi (25) to the response to 1-s lasting stimuli, *in vivo*, supporting the involvement of  $Gd^{3+}$ -sensitive MSCs in the mechanically-induced rapid hyperaemia.

Blockade of mechano-sensitive structures is likely responsible for the decreased vascular reactivity to mechanical stimuli. It is presently not known whether these structures belong to smooth muscle cells as in the classical myogenic response, to the endothelium or both (34). The endothelium is known to release vasodilatory substances, such as NO, prostaglandins and  $K^+$ , upon mechanical stimulation, not limited to shear stress (25, 35, 36). It is particularly interesting to consider the role of  $K^+$  that has been recently shown to have a role in the contraction-induced rapid dilatation (37, 38). In addition to being produced by skeletal muscle fibers during contraction,  $K^+$  may also be released by the endothelium upon activation of endothelial mechano-sensitive TRP channels and then produce hyperpolarization and relaxation of smooth muscle cells through activation of  $K_{ir}$  channels and the  $3Na^+/2K^+$  pump (39, 40). That this pathway may underlie the MC hyperaemic response is also suggested by the recent observation that the dilatatory responses to  $K^+$  (36) and repetitive MC (15) share a transient nature.

On the other hand, about 55% of the response was not affected by  $Gd^{3+}$  at EPC=0.45 mM which suggests that other mechano-transduction pathways, possibly involving non  $Gd^{3+}$ -sensitive MSCs, extracellular matrix - integrins interactions and intracellular adhesion molecules, may concur to the rapid dilatory process (41).

In summary, different pathways may be hypothesized to account for the dual effect of  $Gd^{3+}$  of increasing basal blood flow and decreasing the hyperaemic response to MC. 1) The tonic vasodilator effect may be mediated by the blockade of NSCs in smooth muscle cells. The ensuing hyperpolarization and reduced calcium entry would result in a relaxation of smooth musculature and vessel dilatation (30). 2) The desensitization to mechanical stimulation could result from the blockade of endothelial mechano-sensitive channels resulting in reduced release of vasodilatory factors, presumably  $K^+$ , and reduced vascular dilatations. In addition, other actions of  $Gd^{3+}$  on non mechano-sensitive structures might have contributed to these effects (see below). Further experiments are necessary to test these hypotheses.

A major problem in the administration of  $Gd^{3+}$  (20-50 mg/kg) *in vivo* is related to its toxic effects, namely hypotension, cardiac arrhythmias, A-V bundle blockade and ventricular fibrillation, while no effects are reported below 0.5–1 mg/kg (16). However, with close arterial  $Gd^{3+}$  administration the total amount of drug injected was in the range 0.0017–0.17 mg/kg, *i.e.*, well below the toxic threshold.

In addition  $Gd^{3+}$ , is also prone to precipitate and was observed to lead to the formation of mineral emboli when administered at high concentration (0.14–0.35 mmol/Kg, corresponding to 3.1–7.8 mM plasma concentration) (42, 43). This possibility could not be avoided in the present study and was likely responsible for the sudden reduction of both basal blood flow and of responsiveness to mechanical stimuli observed at EPC of 4.5 mM.

Phenomena like  $Gd^{3+}$  precipitation (42, 43), binding to anions, phosphate, carbonate and EGTA (44), as well as diffusive processes in the interstitium all contribute to reduce the actual  $Gd^{3+}$  concentration as compared to the EPC. This may explain the reason why effects were presently observed at EPC considerably larger than (33) concentrations effective in *in vitro* studies.

Although  $Gd^{3+}$  is considered an effective MSC blocker, largely used in functional experiments on *in vitro* vessel preparation to study the role of stretch-activation cation channels (10, 11, 26, 27), it is not very specific, blocking effects of voltage-gated  $Ca^{++}$ ,  $Na^+$  and  $K^+$  channels having also being reported (16). It cannot be excluded that this side-effects have contributed to the observed increase of basal flow and decrease of rapid hyperaemia. In particular a blockade of calcium channels in smooth muscle cells could have contributed to the observed tonic vasodilatation while a blockade of  $K^+$  channels could have impaired a  $K^+$ -based vasodilatory signalling from the endothelium, thus contributing to attenuate the dilatory response to MC.

The results suggest that  $Gd^{3+}$ -sensitive MSCs take part in the maintenance of basal vascular tone and to the mechanically-induced rapid dilatation of muscle blood vessels. The fact that the hyperaemic response is only partly attenuated by  $Gd^{3+}$  suggests that other mechano-sensitive structures may also be involved.

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