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INACTIVATION OF MECHANO-SENSITIVE DILATATION UPON REPETITIVE MECHANICAL STIMULATION OF THE MUSCULO-VASCULAR NETWORK IN THE RABBIT

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Mechano-sensitivity of the vascular network is known to be implicated in the rapid dilatation at the onset of exercise, however, it is not known how this mechanism responds to repetitive mechanical stimulation. This study tests the hypothesis that the mechanically-induced hyperaemia undergoes some attenuation upon repetitive stimulation. Muscle blood flow was recorded from 9 masseteric arteries (5 right, 4 left) in 6 anesthetized rabbits. Two mechanical stimuli, masseter muscle compression (MC) and occlusion of the masseteric artery (AO), were provided in different combinations: A) repeated stimulation (0.5 Hz, for 40 s); B) single stimuli delivered at decreasing inter-stimulus interval (ISI) from 4 min to 2 s, C) single AO delivered before and immediately after a series of 20 MCs at 0.5 Hz, and vice-versa. Repetitive AO stimulation at 0.5 Hz produced a transient hyperaemia (378 ±189%) peaking at 4.5 ±1.4 s and then decaying before the end of stimulation. The hyperaemic response to individual AOs progressively decreased by 74 ±39% with decreasing ISI from 4 min to 2 s (p<0.01). Non significant differences were observed between AO and MC stimulation. Decreased response to AO was also provoked by previous repetitive MC stimulation, and vice-versa. The results provide evidence that the mechano-sensitivity of the vascular network is attenuated by previous mechanical stimulation. It is suggested that the mechano-sensitive dilatory mechanisms undergoes some inactivation whose recovery time is in the order of a few minutes.

K e y   w o r d s : *exercise hyperaemia, reactive hyperaemia, myogenic response, mechano-sensitivity, muscle blood flow*

INTRODUCTION

The rapid hyperaemia at the onset of exercise, first reported by Gaskell (1), has been the object of intensive research over several decades (2-6). In recent years increased evidence has been gathered supporting the involvement of a mechano-sensitive dilatory mechanism besides the probably slower metabolic mechanisms and the direct propelling action of the "muscle pump" (6-9). The idea was first introduced by Mohrman and Sparks (10) who showed that the hyperaemic response produced by a brief electrically-evoked contraction of an isolated muscle could be partly reproduced by a brief external compression of the muscle. On this basis the hypothesis that the hyperaemic response could result from a dilatory response of the vascular network to the transient decrease in transmural pressure was developed as originally suggested by Bayliss (11). In a recent study, based on continuous monitoring of muscle blood flow *in vivo*, we provided further support to this hypothesis by showing that the hyperaemia produced by a brief spontaneous contraction could be reproduced by a 1-s lasting compression of the relevant muscle, as well as by a 1-s lasting occlusion of the supplying artery (12). This latter manoeuvre, presents the interesting feature of producing a transient decrease in transmural pressure of the muscular-vascular network, while excluding the involvement of both metabolic mechanisms and muscle pump (12).

However, while it has been well documented that a single mechanical stimulation can mimic the rapid hyperaemia induced by a single rapid contraction (7, 10, 12-15) the effects of repetitive stimulation are equivocal as the few studies available in the literature provide conflicting indications. Repetitive external compression was observed to slightly reduce the hyperaemic response in the human forearm, although the effect did not reach statistical significance (7), while the same pattern of stimulation enhanced the dilatory response of isolated arteries suggesting the presence of a potentiation process (8).

In the former study (7) the extent of perfusion during and in between stimuli was not reported, possibly due to the low time resolution of the blood flow measurement (1 value per heart beat). In addition, in both the above quoted studies (7, 8), the series of stimuli was limited to five consecutive compressions while the effect of a longer lasting mechanical stimulation was not tested. Few previous studies did investigate the effect of long-lasting repetitive compression of the forearm, however, in such studies the attention was focused on the role of the muscle pump in the steady state rather than on early blood flow transients (3, 15), thus leaving the issue unsettled. However, the observation that in the steady-state (*i.e.* the stable perfusion level that is reached before the end of the stimulation) little or no blood flow increase was reported (3, 15) led us to hypothesize that an "adaptation" rather than a potentiation occurs in response to continuous mechanical stimulation.

In order to test this hypothesis, the hyperaemic response to repetitive mechanical stimulation is here investigated utilizing a previously validated experimental model in which blood flow is recorded from the exclusively-muscular masseteric artery, in the anesthetized rabbit (16). As compared to monitoring blood flow from large mixed arteries, this feature excludes the unresponsive cutaneous blood flow from the measurement (12), thus granting a higher sensitivity for the detection of hemodynamic responses to mechanical stimuli. In addition, this experimental setup allows for the direct and continuous monitoring of flow during and between mechanical stimuli, throughout a long lasting stimulation. Two types of stimuli are adopted, *i.e.*, muscle compression (MC) and occlusion of the supplying artery (AO), the latter allowing us to exclude the involvement of the muscle pump (12). Three protocols were implemented in order to 1) describe the response to repetitive stimulation, 2) investigate the dependence of the hyperaemic response to MC and AO on the inter-stimulus interval and 3) investigate whether preconditioning the vascular network with repetitive AO or MC could affect the response to MC and AO, respectively. This latter approach was implemented to confirm or disprove the assumption that the responses to MC and AO indeed result from the same dilatory mechanism. On this basis, the responses to MC and AO were expected to be similarly affected by reciprocal preconditioning with repetitive stimulation.

MATERIAL AND METHODS

Ethical approval

The study was performed at the University of Torino 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 Torino.

Surgical procedure

Experiments were carried out on 6 male European rabbits (Oryctolagus cuniculus) weighing between 2.9 and 3.3 kg, anesthetized with urethane (Urethane Sigma Aldrich dose 1.2 g kg-1 i.v.). Full surgical anaesthesia was maintained by injecting additional doses of the drug (0.4 g kg-1 i.v,) through a catheter inserted in the cannulated left femoral vein. In all animals trachea was cannulated and the head was fixed in a stereotaxic frame by screws implanted in the nasal and frontal bones.

A telemetric blood pressure transducer (TA11PA-D70, DSI USA) was implanted, the catheter being inserted into the right femoral artery.

A perivascular flow probe (model 0.7PSB, Transonic Systems Inc, Itaha, NY, USA) was implanted on the masseteric branch of the facial artery (Ma), as previously described (16). In 3 rabbits the flow probe was bilaterally implanted so that 9 arteries were investigated. The experimental procedures started after stabilization of the hemodynamic variables, about 1 h 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.

Experimental protocol

While blood flow was recorded from the Ma, hyperaemic responses were evoked by mechanical compressions (MC) of the masseter muscle and by artery occlusion (AO). MCs were performed by means of a servo controlled motor (mod 310B Level System, Cambridge Technology,Inc) pushing a cylindrical head (diameter 1.3 cm) against the anterior part of the cheek. The motor is driven by a personal computer through a D/A converter (1401 micro, CED, UK) and the timing of the stimulus is precisely determined by the supplied analog signal which drives the displacement of the cylindrical head towards the muscle. The force exerted on the muscle is however "clipped" by a maximum torque level manually set on the motor control unit. In this way, the force (pressure) level is maintained strictly constant during each stimulus and across subsequent stimuli. In a previous methodological investigation we observed that hyperaemic responses to MC were little dependent on the pressure level (17). Based on this previous experience we adopted a moderate force level of 1.8 N, resulting in a locally exerted pressure of about 100 mmHg. The trapezoidal stimulus is characterized by a plateau duration of 1 s and rising and falling time of 0.1 s.

The AO lasting about 1 s was performed by the experimenter by means of a moderate compression of the skin surface exerted by a finger pressed against the inferior margin of the mandible at the point where the masseteric artery passes over the bone (downstream to the flow probe) as previously described (12). Effectiveness of the manoeuvre was confirmed by the interruption of blood flow in the masseteric artery. Vascular reactivity was investigated in response to three different stimulation protocols pursuing three different aims, as described below and illustrated in *Fig. 1*: *A*) a series of 20 mechanical stimuli (AO or MC), with inter-stimulus interval (ISI) =1 s, with the aim of describing the response to a standard repeated stimulation; *B*) a series of 6 equally-spaced stimuli (AO or MC) repeated 8 times, each time with a progressively decreased ISI as follows: 240, 120, 60, 30, 16, 8, 4, 2 s, with the aim of investigating the dependency of the response on the ISI; C) a series of 20 mechanical stimuli (AO or MC) was preceded and immediately followed by a single stimulus of different type (MC or AO). An interval of 240 s separated the first stimulus from the series and an interval of about 8–10 s separated the series from the second single stimulus. This latter protocol is aimed to investigate whether preconditioning the vascular network with one stimulus would affect the response to the other „stimulus".

Data acquisition and processing

The arterial blood pressure signals were radio-transmitted from the implanted transducer to a nearby located antenna and reconverted to an analogue voltage signal (TA11PA-D70, DSI USA) while the flow probes were connected to the flow meter (2-channels TS420, Transonics, USA) *via* 1.5 m extension cables. All signals were then digitally sampled (1401micro, CED, UK) (sampling rate: 200 Hz) and continuously acquired and stored on a personal computer. Acquisition and off-line processing were performed with Spike2 (CED, 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. *Fig. 2* shows a representative example of the hyperaemic response to MC and AO and shows how the response was characterized in terms of maximum blood flow increase (peak amplitude) and time elapsed from the beginning of the response to the flow peak (time-to-peak).

A) Repetitive stimulation at constant rate

The time course of the hyperaemic responses to repetitive stimulation was described by measuring the average flow in-between subsequent stimuli (OFF phase). The initial 0.1 s of the OFF phase were not included in the analysis in order to exclude the rapid flow transients, occurring immediately after the release of the occlusion and attributed to vascular compliance (see also

Fig. 2B). In addition, the actual muscle perfusion was assessed by averaging blood flow over the whole OFF-ON cycle.

B) Stimulation at increasing rate.

Hyperaemic responses were normalized with respect to the basal flow recorded before the beginning of the whole „series". Peak flow was measured from the response to each stimulus in the sequence (6 stimuli * 8 stimulation frequencies = 48 stimuli). Within each 6-stimuli sequence performed at a given ISI, the response was shown to stabilize after 1–2 stimuli. Therefore computation of average peak flow was limited to the last 4 stimuli in the sequence. The value collected at each stimulation frequency was then compared to the value collected at ISI =4 min, in order to assess the dependence of peak flow with the ISI.

C) Single stimulation before/after conditioning.

We have here analyzed the changes in hyperaemic response to the single AO and MC stimuli after preconditioning the vascular network by repeated stimulation with the MC and AO, respectively. Changes were assessed both in terms of peak amplitude and of area-under-the-curve, computed with respect to the control level measured before the repeated stimulation.

Statistics

In protocol A, the responses to repeated stimulation with MC and AO were compared with a 2-way ANOVA, with factors time (20 levels) and stimulus type (2 levels: MC, AO). The Dunnett post-hoc test was employed to assess significance of blood flow increase with respect to control level. A 2-way ANOVA and Duncan post-hoc test, was also adopted to compare the time course of the response to repetitive versus single stimulation.

In protocol B, the effects of stimulus type and inter-stimulus interval (8 levels) were assessed by a 2- way ANOVA and Dunnett post-hoc test for the 2 parameters: peak amplitude and basal blood flow.

In protocol C, the effect of time (before/after repeated stimulation) and of stimulus type (MC/AO) were also tested by a 2-way ANOVA and Duncan post-hoc test..

All measures are reported as mean ±standard deviation.

RESULTS

In resting conditions, blood flow in the masseteric artery, ranged between 0.12 and 0.53 ml min-1 (average: 0.29±0.11 ml min-1). Arterial blood pressure remained stable throughout the experiments (123±6 mmHg) and was not affected by mechanical stimulation, *i.e.*, MC and AO. It can be observed that the two manoeuvres elicit the same type of hyperaemic response although blood flow is completely stopped during AO and not during MC (*Fig. 2*).

Repetitive stimulation at constant rate

The hyperaemic response to repetitive stimulation at constant rate was investigated both with MC and AO according to the protocol depicted in *Fig. 1A* (1 s ON, 1 s OFF, for 40 s). *Fig. 3A* and *3B* show representative examples of the response to repetitive MC and AO, respectively. The hyperaemia increases rapidly, reaching maximum between the second and the fourth stimulus and then slowly decays in spite of continuing stimulation, for both manoeuvres. After the end of stimulations blood flow gradually returns, never decreasing below control levels.

Average hyperaemic responses to repetitive stimulation were first computed considering blood flow levels in-between stimuli (OFF-phase; *Fig. 4A*, black traces). It can be observed that responses are almost overlapping with no significant difference between MC and AO (p=0.64) but with a significant dependence on time (p<0.01). Blood flow remaining significantly elevated with respect to control until the end of stimulation (MC: +97 ±75%, p<0.05; AO: +140 ±134%, p<0.05).

Fig. 4A also shows, for comparison the hyperaemic response to a single stimulus (grey traces). Peak amplitude was not
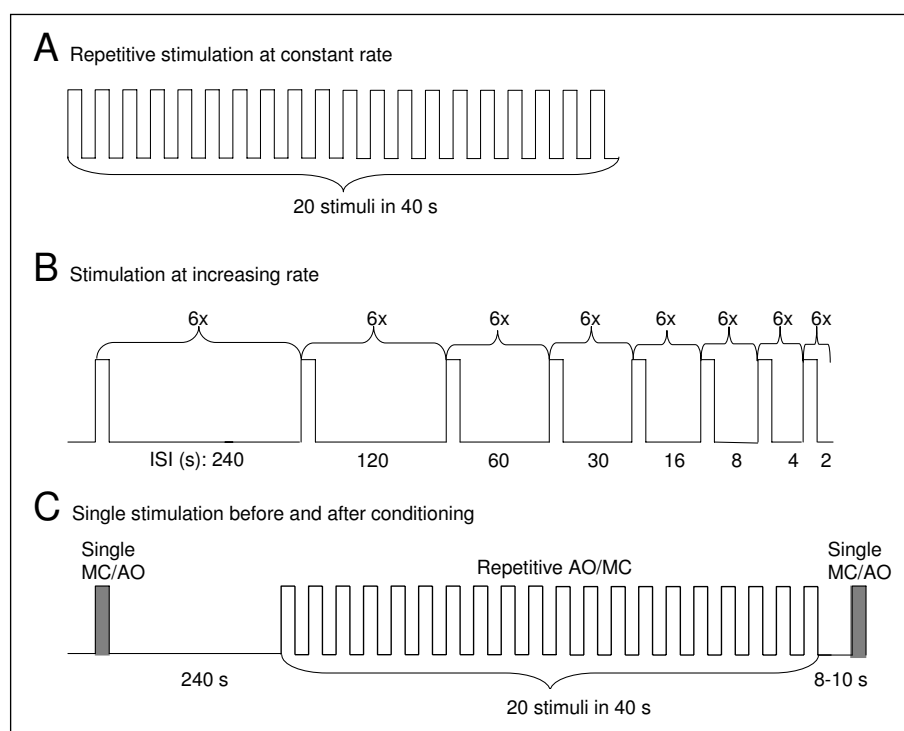

Fig. 1. Experimental protocols. Mechanical stimulation by muscle compression (MC) or artery occlusion (AO), both lasting 1 s, is provided according to 3 different protocols: *A*) stimulation at a constant rate of 0.5 Hz for 20 s; *B*) stimulation at increasing rate: the inter-stimulus interval (ISI) is progressively decreased from 240 to 2 s in 8 discrete steps performed every six stimuli; *C*) Single stimuli (MC or AO) are provided before and few seconds after a repetitive stimulation (rate: 0.5 Hz, duration: 20 s) of different type (AO or MC, respectively).
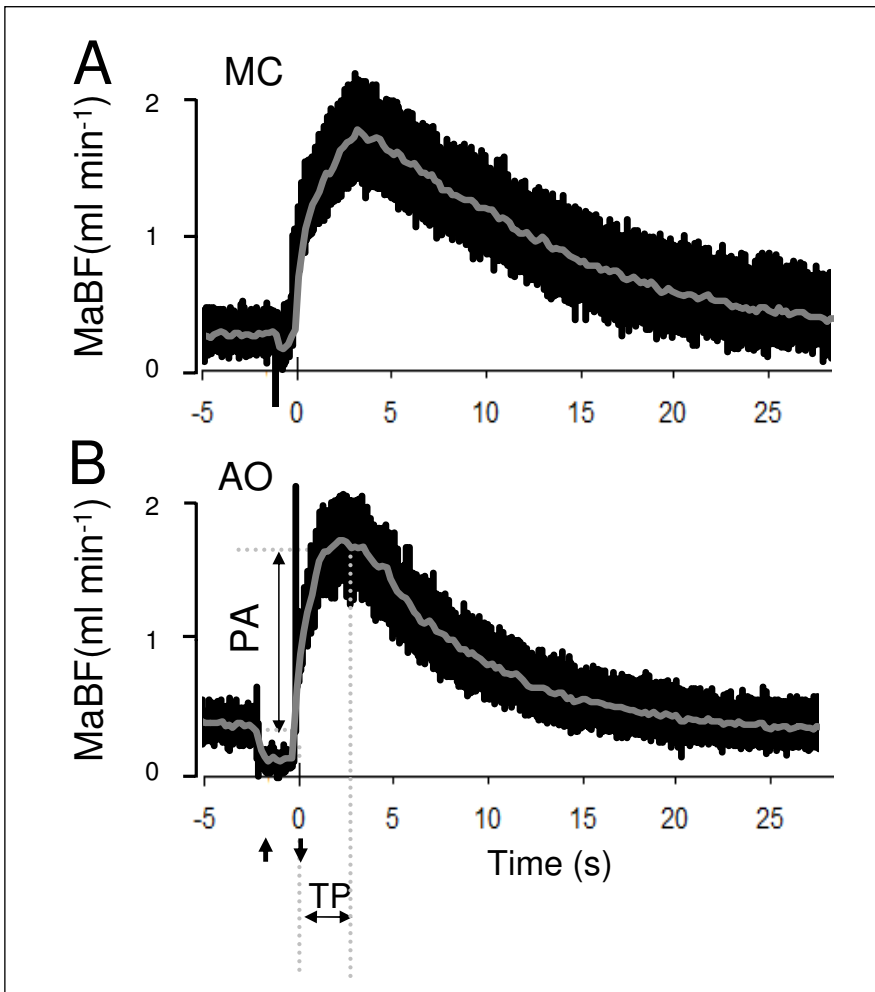
Fig. 2. Examples of hemodynamic responses to single stimuli. Blood flow of the masseteric artery is recorded in response to 1-s lasting compression of the masseter muscle (*A*) and to 1-s lasting occlusion of the masseteric artery (*B*). Note that complete occlusion of blood flow during the stimulus occurs only in *B*. Time-averaged blood flow (grey) is superimposed on the original unfiltered recording (black). MC - muscle compression; AO - artery occlusion; PA - peak amplitude; TP - time-to-peak. The arrows indicate onset and termination of the stimulus.

significantly different in the different curves: single MC: 429±228%; repetitive MC: 403 ±178%; single AO: 477 ±179%; repetitive AO: 378 ±189%. However, the response to repetitive stimulation exhibited a slower decline than the response to the single correspondent stimulus, significant differences being marked with asterisks for both MC (*Fig. 4A*, thick lines) and AO (thin lines).

The actual increase in perfusion was assessed by considering the average blood flow computed over the whole OFF-ON cycle (*Fig. 4B*). Due to the complete blood flow occlusion during the ON phase of AO but not of MC, the AO trace (thin line) results considerably dampened compared to MC, although ANOVA did not evidence a dependence on stimulus type (p =0.13). The responses were dependent on time but residual hyperaemia at the end of the stimulation (AO: +51 ±61%; MC: +55 ±50%) was not significantly different from control blood flow.

Stimulation at increasing rate

With the second stimulation protocol (*Fig. 1B*) we investigated how the hyperaemic response to a single mechanical stimulus is affected by the inter-stimulus interval.

At each step-increase in stimulation rate (decrease in ISI) the amplitude of the hyperaemic response quickly stabilized, at a progressively lower level. A representative example of the response to MC at different stimulation frequencies is shown in *Fig. 5A*, while the average pattern of response to the whole sequence of stimuli is reported in *Fig. 5B* for MC and in *Fig.*

5C for AO. The normalized peak flow was statistically dependent on the interstimulus interval (p<0.01) but not on the type of stimulus (p=0.77). At ISI =240 s (4 min) peak flow was 662 ±170% of resting blood flow in response to MC and 620 ±205% in response to AO. With respect to this maximum response, significant changes at higher stimulation frequencies are indicated by asterisks in *Fig. 5B* and *5C*. Peak blood flow significantly decreased to 600 ±289% and 525 ±70% at ISI =60 s, to 457 ±170% and 439 ±108% at 30 s and to 209 ±91% and 233 ±231% at 2 s, for MC and AO respectively (p<0.05).

Note that the response observed at (ISI =2 s) overlaps the response observed at the end of protocol A (ISI =2 s from the beginning of the stimulation) which confirms the validity of protocol B to assess the steady-state response at the different ISIs.

With respect to ISI = 240 s the peak amplitude of the response was reduced by 11% and 18% at ISI = 60 s, by 36% and 34% at 30 s, and by 81% and 74% at 2 s, for MC and AO, respectively.

In addition a slight reduction in basal blood flow was observed in-between hyperaemic responses which depended on ISI (p<0.05) and not on the type of stimulus. This effect was absent at ISI =4 min and increased with stimulation rate reaching a maximum of 14 ± 20% (p<0.05) (reduction with respect to pre-stimulation level) at ISI = 30 s and of 11 ± 22% at ISI=60 s (p<0.05). At higher rates the effect could not be assessed due to overlapping of hyperaemic responses.
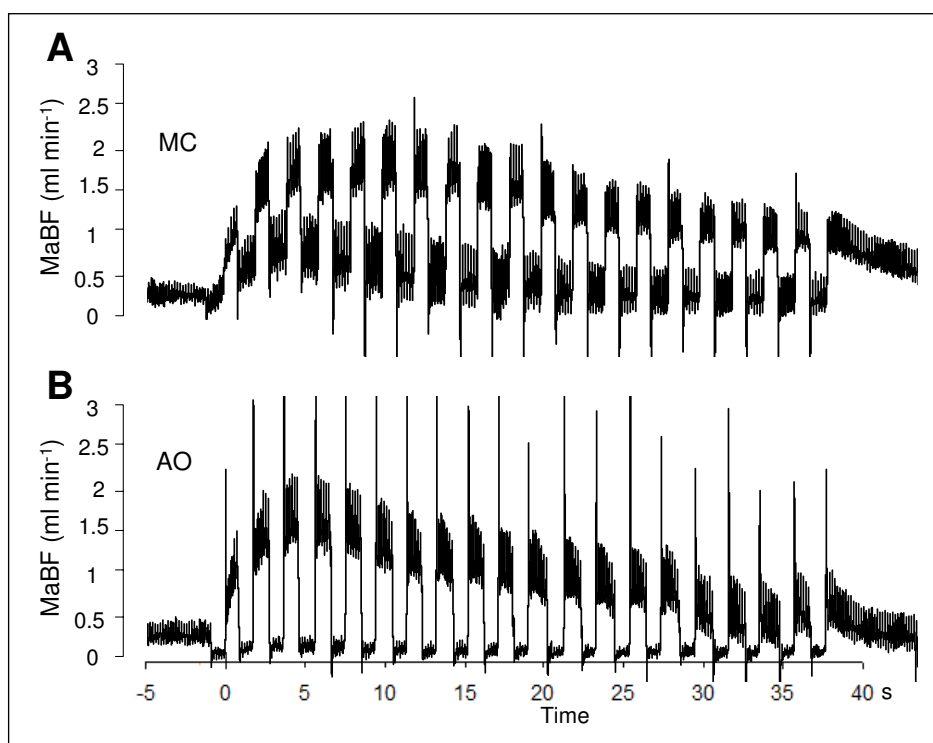
Fig. 3. Representative examples of the hyperaemic response to repetitive stimulation, according to protocol of *Fig. 1A*.
A) muscle compression (MC), *B*) artery occlusion (AO). The recordings are collected from the same artery. Note in both cases the progressive decrease in blood flow in spite of continuing stimulation. Time = 0 s at the release of the first stimulus. MaBF - masseteric artery blood flow.
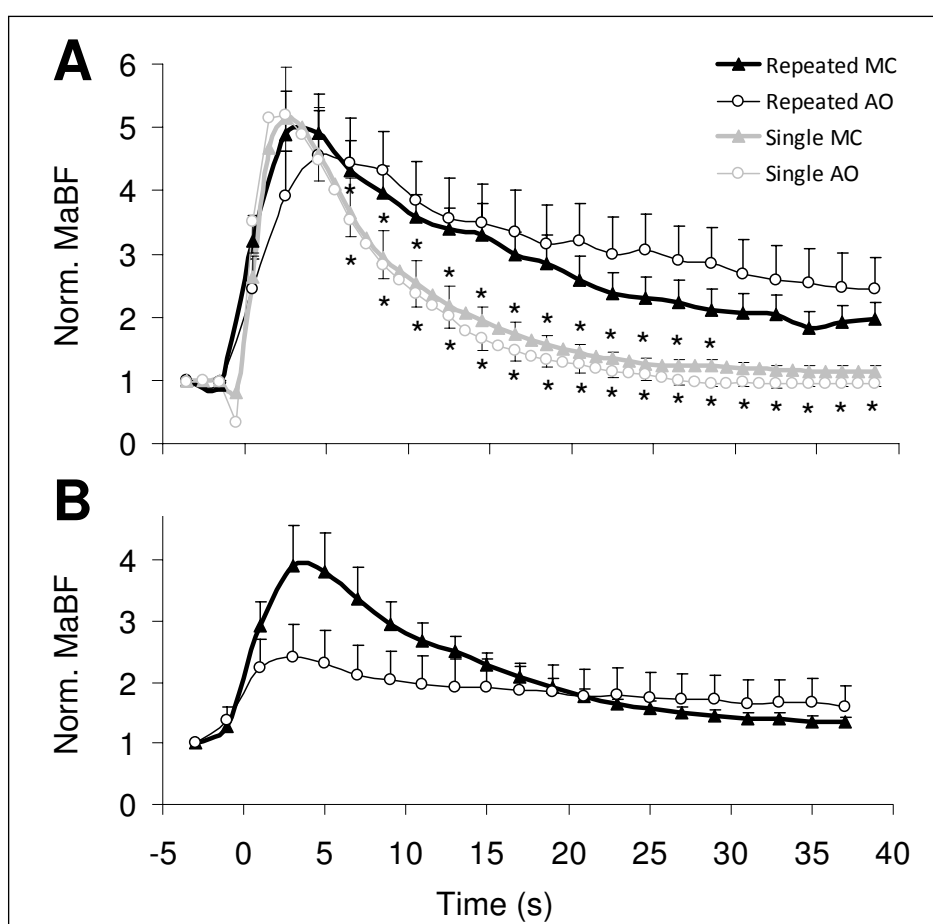

Fig. 4. A) Hyperaemic response to repeated stimulation (protocol of *Fig. 1A*) with AO (thin line, circles) and MC (thick line, triangles). Blood flow is measured during the OFF phase (*i.e.*, in-between stimuli). For comparison, average response to single AO (thin grey line) and MC (thick gray line) are also reported.
B) Actual muscle perfusion during repeated stimulation, evaluated by measuring average blood flow over the whole OFF-ON cycle. Responses were individually normalized to resting blood flow level (n = 9). Error bars represent standard error. Time = 0 s at the release of the first stimulus. Abbreviations as in *Fig. 3*. *- Significantly different from response to repetitive stimulation (same stimulus): $p < 0.05$.

Single stimulation before/after conditioning

By means of the protocol presented in *Fig. 1C* we sought to verify whether the response to a single MC or AO could be modified by previous conditioning produced by repetitive stimulation of different type, *i.e.*, AO or MC, respectively.

Fig. 6A reports the original recording of the responses to a single MC performed before and few seconds after repetitive
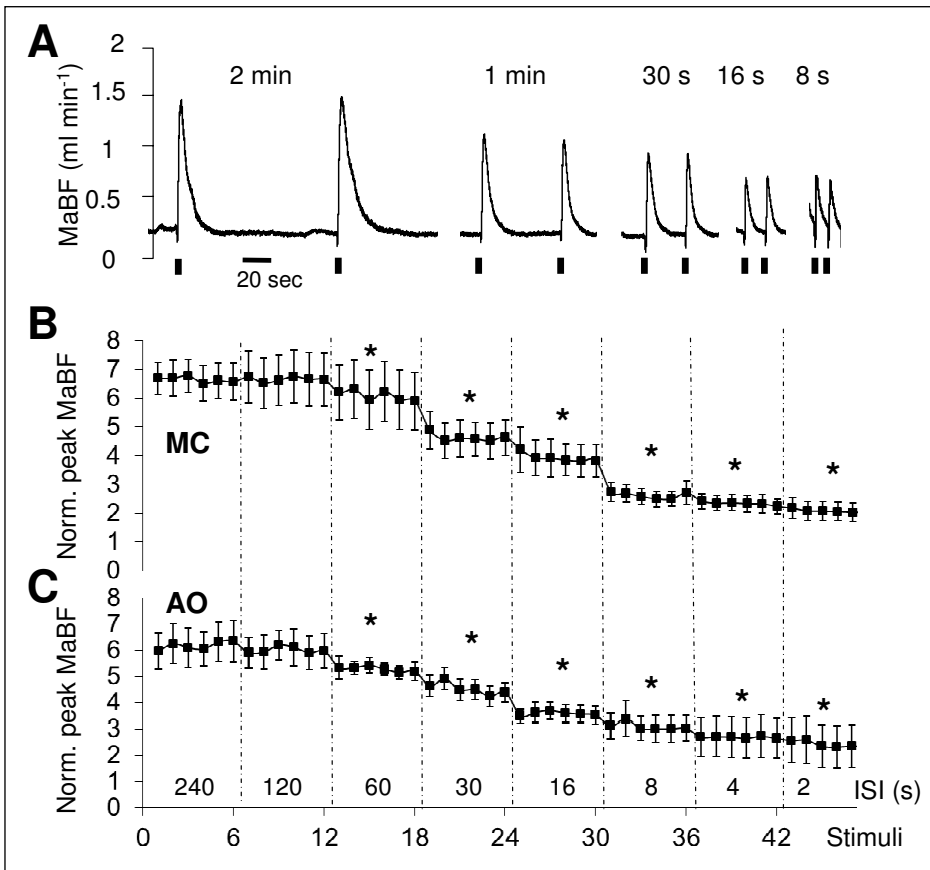
Fig. 5. A) Original recordings of the hyperaemic response to muscle compression at increasing rate, according to protocol of *Fig. 1B*. For the sake of readability responses to selected pair of stimuli at different ISI have been presented. Below the recordings the black marks indicate the time of occurrence of the stimuli and the label above indicates the ISI. *B*) and *C*) The peak flow of the response to MC (*B*) and AO (C) is reported for every single stimulus of the protocol. The ISI, maintained constant within the groups of 6 stimuli delimited by dash-dot lines, is indicated by the labels above the X-axis while the labels below the axis indicate the ordinal number of stimuli. Asterisks indicate significant decrease of peak flow (average of last 4 responses in each group) with respect to the maximum response at ISI = 240 s, (p<0.05). Responses were individually normalized to resting blood flow level (n = 9). Error bars indicate standard error. Abbreviations as in *Fig. 3*.

AO. It can be observed that the response to MC is considerably reduced after conditioning. Similarly, the response to a single AO is considerably attenuated by previous conditioning with a repetitive MC as shown in *Fig. 6B*. Average effects are displayed in the bar diagrams of *Fig. 6C* and *6D* for the two protocols, in terms of peak amplitude and area-under-the-curve of the responses to the single stimulus before and after conditioning. Conditioning by repetitive MC reduced the amplitude of the AO response by 62 ±29% (p<0.01) and the AUC by 82 ±30% (p<0.01). Conditioning by repetitive AO reduced the amplitude of the MC response by 78 ±15% (p<0.01) and the AUC by 91 ±7% (p<0.01).

DISCUSSION

The present study yielded the following new findings 1) repetitive mechanical stimulation does not increase blood flow beyond the level obtained with a single stimulus. On the contrary, the hyperaemic response exhibits a progressive attenuation in spite of continuing stimulation, which commences 4–6 s after stimulation onset (*Fig. 3* and *4*); 2) attenuation is also observed in the hyperaemic response to single stimuli delivered at progressively decreasing ISI: peak amplitude decreased over 70% at ISI =2 s, as compared to ISI =4 min (*Fig. 5*); 3) the above data are observed independently of the type of mechanical stimulus delivered, MC or AO, and the response to AO is attenuated by previous repetitive MC stimulation and vice-versa (*Fig. 6*).

Few studies have investigated the vascular response to repeated mechanical stimulation. In a recent study, Kirby *et al.* (7) reported a non significant reduction of the response to repeated compression of the forearm. As compared to the single

compression (200 mmHg for 2 s), the response to 5 consecutive compressions (assessed after the 5th stimulus) was attenuated by an estimation of about 26%, although the effect did not reach statistical significance, and had the peak anticipated from the 2nd to the 1st cardiac cycle (post compression) (7). These effects are explained by the present results, which show in fact that the hyperaemia progressively decreases upon continuous stimulation (peak response after the 5th MC is 23% lower than the response to a single MC, *Fig. 4A*, thick lines). In addition, assessment of blood flow in-between stimuli allowed us to evidence that, after the first 3–4 stimuli, the peak hyperaemia is almost immediately reached at the release of each of the subsequent stimuli (*Fig. 3A* and *3B*); this indicates that the vascular bed is already dilated and that subsequent stimuli give little additional contribution (discussed below).

The same pattern of 5 consecutive compressive stimuli was also tested on isolated muscle feed arteries resulting instead in increased dilatory response, as compared to the single compression (8), which is in apparent conflict with the present study. On the one hand, this difference could be attributed to the absence of autoregulatory metabolic mechanisms in the *in vitro* model (discussed below). On the other hand it is possible that the higher pressure stimuli employed in such study (600 mmHg) have recruited additional mechano-sensitive mechanisms that do not exhibit inactivation. In addition, the different results may be explained by a different reactivity of feed arteries, as compared to intramuscular vessels (18), which are instead the major recipients of the transmural pressure change produced by AO and MC in the present study. Investigation of the effect of longer sequences of stimuli on isolated vessels will be necessary to clarify this issue.

The possibility to exclusively monitor muscle blood flow granted high sensitivity to the present experimental model
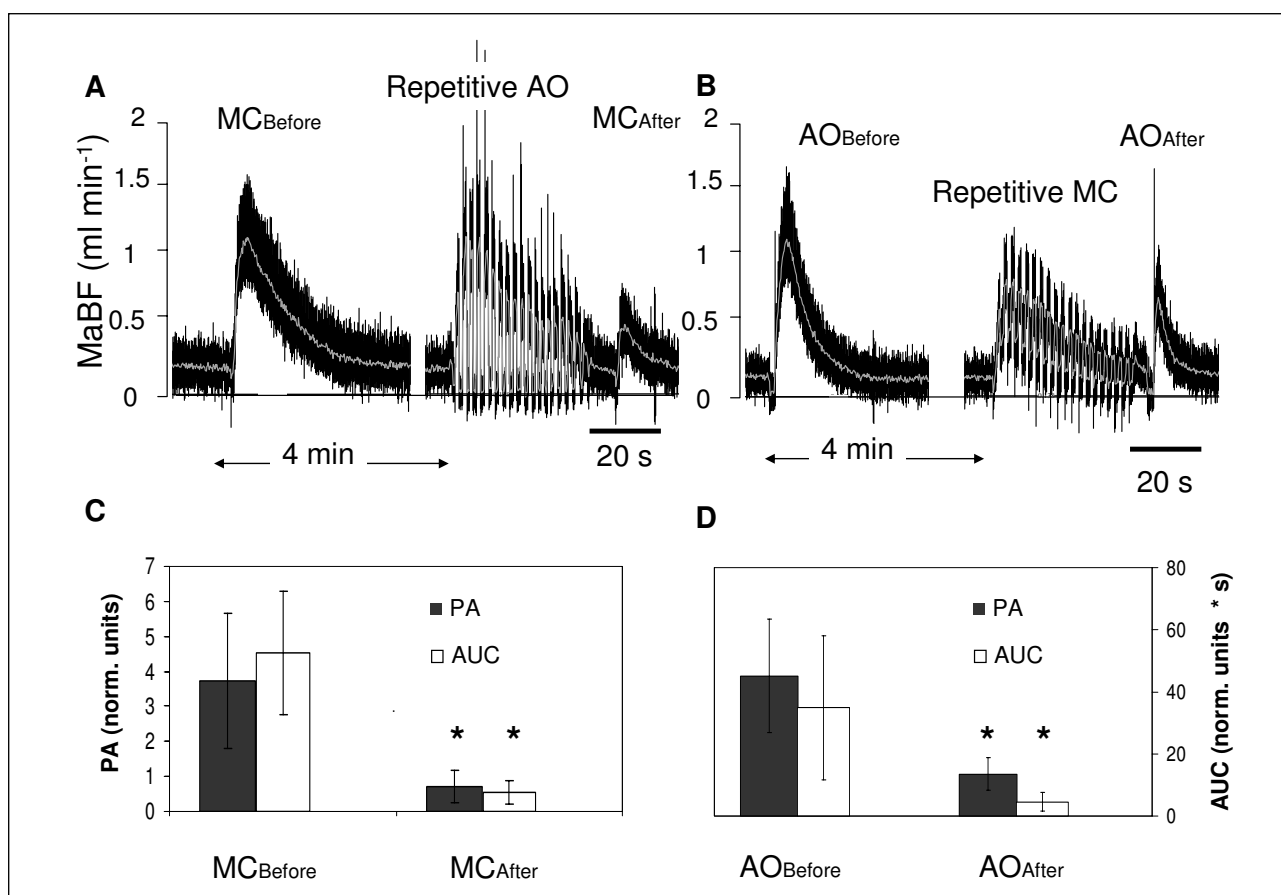
Fig. 6. Response to muscle compression is attenuated by previous repetitive artery occlusion (*A*) and vice versa (*B*), according to protocol of *Fig. 1C. C*) Bar diagrams indicating the attenuation of the response to MC after repetitive AO. *D*) Bar diagrams indicating the attenuation of the response to AO after repetitive MC. Amplitude of the response is assessed both in terms peak amplitude (PA, black bars, normalized to resting blood flow level) and of area under the curve (AUC, white bars). Abbreviations as in *Fig. 3.*

allowing to detect a clear cut increase in perfusion also in response to repetitive AO (*Fig. 4B*). This demonstrates that the muscle pump mechanism is not strictly necessary to the initial hyperaemia and its subsequent attenuation, although it may contribute to a sustained blood flow increase during repeated compressions or contractions (3). In this respect, it is interesting to recall the study by Tschakovsky *et al.* (3) who investigated the effect on brachial blood flow of repeated forearm compressions with the arm kept either below or above heart level. In the first condition they observed a response similar to the one obtained with protocol A (*Fig. 4*), *i.e.*, a rapid increase in blood flow that, after a first peak (+60% of control), stabilized at about 35% above control level. Interestingly, a transient hyperaemia could be noticed also with arm below heart level (their *Fig. 1*), although the effect did not reach significance (3). That study helped to understand the role of the muscle pump but its results also suggest that the transient nature of mechanically-induced dilatation described in the present study concerns also human muscles.

We did not find in the literature other reports on attenuation of the mechano-vascular sensitivity to repeated stimulation, with the exception of a brief reference to a "myogenic fatigue" (19) observed in isolated human umbilical artery (20). In that study the artery was subjected to periodic increases in transmural pressure obtained by transiently decreasing the extra vascular pressure (−80 mmHg, for 10 s, repeated every minute). In some of the arteries the constrictor response to the mechanical

stimulus progressively decreased until complete exhaustion. No decrement in the response was instead observed if the interval between subsequent stimuli was increased to 10–20 min (20). Thus, although the effect is characterized by a different time course and relates to a different experimental model, it is interesting to observe that a qualitatively similar reduction of the vascular response upon repetitive mechanical stimulation may affect both the rapid dilatory and the classical ("myogenic") constrictor response.

Two possible mechanisms may be envisaged to explain the attenuation of the hyperaemic response to repetitive stimulation: 1) the perfusion/metabolism mismatch, taking place during the hyperaemia produced by prior stimulation, activates local metabolic mechanisms that counteract further dilatory stimuli, as suggested by Tschakovsky *et al.* (3); 2) the mechano-sensitive mechanism or the biochemical pathways involved in the dilatory response are inactivated by repetitive stimulation.

Possible role of local autoregulatory mechanisms

If hyperaemia occurs in a condition in which local metabolism is unaltered, the excess of blood flowing through the muscle potentially alters tissue homeostasis mainly in terms of PO_2, PCO_2 and pH and these changes would trigger, in turn, a vasoconstrictor response. A role for such autoregulatory mechanisms is suggested by the observation that muscle perfusion returns to basal levels before the end of repeated

stimulation (*Fig. 4B*). However the timing and extent of the response to hypeperfusion in skeletal muscle are not precisely known (21).

Information on the specific role of blood gases in the control of vascular tone is provided by studies investigating the effect of altering the concentration of O_2 and CO_2 in arterial blood. Selective increase in arterial O_2 partial pressure to about 700 mmHg produces only slight (10–20%) reduction in resting muscle blood flow (22-25), while the local reaction to hypocapnia (under blockade of sympathetic efference) appears to be a dilatation (26, 27).

Interestingly, few studies also investigated the effects of altered PO_2 and PCO_2 on reactive hyperaemia (22, 28). We here remind that the reactive hyperaemia, *i.e.*, the hyperaemic response to transient occlusion of the arterial supply, is considered to result from a mechano-sensitive mechanism, namely the myogenic response to the transient decrease in transmural pressure, for occlusion durations up to 30 s, while metabolic mechanisms would play a role only in response to longer lasting occlusions (19, 29-33). This pattern of response, which could result also from the involvement of the endothelium (31), is adequately evoked also by very short (1 s) lasting occlusions, such as the AO stimulus adopted in the present and in other studies (12, 30). Thus, it is interesting to observe that peak flow in reactive hyperaemia is reported to be unaffected or only slightly reduced by PO_2 increases (22, 32, 34), PCO_2 decreases (22) and by muscle perfusion with venous blood (28). In particular, Tuma *et al*. (34) showed that exposure of the tenuissimus muscle to a hyperoxic superfusate (150 mmHg PO_2) did not significantly affect the peak hyperaemic response to 120-s lasting artery occlusions in spite of a 75% reduction in resting blood flow.

On the contrary, in the present experiments resting blood flow was only slightly affected (–13%, in-between stimuli at ISI = 30 s) while peak amplitude of the AO response was shown to decrease by 34% at ISI = 30 s and to 81% at ISI = 2 s. Thus, it seems unlikely that this marked desensitization to mechanical stimuli, unaccompanied by prominent constrictor effects, can be mediated by autoregulatory mechanisms triggered by the short lasting hyperaemic response to preceding stimuli.

Inactivation of the mechano-sensitive mechanism

Can the desensitization of the vascular network to mechanical stimuli result from the inactivation of the mechano-sensitive mechanism or of the involved biochemical pathway? The hypothesis is not unrealistic since desensitization and inactivation mechanisms are frequently observed in mechano-sensitive channels (35-37), which in turn are considered among the most likely mediators of mechano-sensitivity in vascular smooth muscle and endothelium (38, 39). For example, an *in vitro* investigation reported desensitization of TRAAK channels (a sub-type of 2-pore-domain potassium channels) upon mechanical stimulation, which was attributed to inactivation mechanisms (36). The described pattern of inactivation (their *Fig. 2C*) is remarkably similar to the one shown in present *Fig. 3* and *4*, except for developing on a shorter time scale (recovery time of about 1 s).

By comparing the hyperaemic response to single and repeated stimulation in *Fig. 4A*, we can observe that the second mechanical stimulus, occurring 1 s after the end of the first one (in protocol A), fails to provide any additional increment in the hyperaemic response and the same applies to the next few stimuli. In fact, only after the 4th stimulus the hyperaemic response to repeated stimulation becomes significantly higher than to the single stimulus (*Fig. 4A* and *4B*). In principle, this phenomenon could be explained by

saturation of the dilatory process. However, it seems unlikely that complete saturation is already achieved after the very first stimulus in the series. Moreover, this would not explain the decline of the hyperaemia in spite of continuing stimulation, commencing as early as 7–8 s from the beginning (protocol A). Finally it was observed that a 1-min interval was still not enough for a complete recovery of the vascular mechano sensitivity, the response at ISI = 1 min being significantly smaller than at 4 min (protocol B, *Fig. 5*).

On this basis, a plausible explanation for the observed effects is that a rapid inactivation of the mechano-sensitive mechanism takes place after the first stimulus, with a recovery time in the order of a few minutes. Besides this possibility, the progressive attenuation of the response to mechanical stimulation could be mediated by the involved dilatory pathway. One candidate is nitric oxide since it is known to be released by endothelium in response to mechanical stimulation, which could directly derive from the applied stimulus or from the increased shear stress during the ensuing hyperaemia (31, 40, 41). However, several studies excluded a major role of NO in mechanically-induced rapid dilatation (8, 13, 31). Local plasma [ATP] was recently found to increase after repeated muscle compression (42, 43) and was suggested to contribute to functional hyperaemia. However, both [ATP] and blood flow were assessed after several minutes (4–7 min) of repeated compression of forearm or thigh muscles (42, 43) and their time course from the beginning of the stimulation is not known. An intriguing possibility is constituted by prostaglandins that appear to be an important mediator of the mechanically-induced rapid hyperemia. Blockade of prostaglandins was found to reduce by 74% the response to 1-s lasting AO in the dog hind limb (44). More recently, the hyperaemic response to 1-s lasting contraction of the forearm was also found to be reduced by 27–34% after combined NO-prostaglandin blockade (45), indirectly supporting the concept that the rapid dilatory responses to AO and contraction share the same underlying mechanisms (12). Interestingly, the hyperaemic response to repeated muscle contraction (rhythmic handgrip at 20% MVC, for 4 min) in the same experimental model was not affected by the same combined blockade (46), which would be compatible with the idea of mechano-sensitive dilatory mechanisms that fade away upon repeated stimulation. Finally, K^+ has been shown to play a role in the rapid dilatation induced by a short lasting contraction (45, 47). The similarity in the response to active contraction and mechanichal stimulation of the passive muscle (7, 12) suggest that K^+ of skeletal muscle origin is not necessary to the rapid dilatory process. On the other hand K^+ could also be released within the vessel wall (48) upon mechanical stimulation (49). In particular, two features make the involvement of K^+ particularly attractive: 1) K^+-induced dilatation has a transient nature (50, 51) and 2) is prominent in muscular and absent in epithelial arterioles (51), which respectively find good correspondence with 1) the transient hyperaemic pattern described in the present study and 2) the different responsiveness of skin and muscle vascular beds to mechanical stimulation (12).

Although the present study was not designed to investigate the molecular basis of mechano-transduction and the dilatory pathways involved, the here-described desensitization process will possibly provide a cue for the identification of the mechanisms behind the mechanically-induced rapid dilatation.

Functional implications

In a recent study based on this same experimental model the observation was made that AO, MC, passive movement (muscle

stretch) and short-lasting active contraction all produce a comparable rapid hyperaemic response, which lead us to hypothesize that a single mechano-sensitive mechanism, possibly responding to the reduction in vascular transmural pressure (Bayliss effect) could underlie all responses (12). We here observed that a similar desensitization pattern is exhibited by both AO and MC upon repetitive stimulation, the same pattern being also produced by repetitive muscle stretch (unpublished observation). In addition the preconditioning by repetitive AO effectively attenuates the response to MC and *vice versa* (*Fig. 6*). These observations further support the notion that all these mechanical stimuli activate the same mechano-sensitive pathway (12). In this respect, it is interesting to note that a transient hyperaemia lasting 20–30 s, similar to the patterns presented in 4, is consistently observed in response to repetitive passive limb movement (90 degree flexo-extension of the knee at 1 Hz) (52-54). The present results strongly support the notion that passive movement hyperaemia originates from peripheral (54) rather than from central mechanisms (52). Moreover they support the notion that the improved muscle perfusion by passive exercise, as well as by muscle compression, is better achieved through an intermittent treatment than through a prolonged one (53).

Few years ago, Kirby *et al.* (7) first suggested that the mechanically-induced rapid hyperaemia would constitute a feed forward dilatation at the onset of exercise, evoked by the mechanical action of muscle contraction on intramuscular blood vessels. The observation that the muscle exhibits a considerably higher dilatory response to mechanical stimulation, as compared to cutaneous tissue (12), is in agreement with the hypothesis. The present observation of the transient nature of the hyperaemia during repeated stimulation also nicely fits with this concept. At the onset of exercise the muscle benefits of promptly increased perfusion by mechano-sensitive mechanisms and, if the exercise continues, the slower metabolic dilatation will progressively take over. If the ongoing mechanical stimulus is unrelated to muscle activity (as in passive limb movement or external muscle compression) the hyperaemia soon fades away, thus limiting useless muscle hyperperfusion and saving systemic resources.

Conclusions

In the present study evidence is provided that the mechano-sensitivity of the musculo-vascular network, responsible for mediating a rapid hyperaemia, exhibits a desensitization upon repeated stimulation. Although the involvement of metabolic mechanisms cannot be completely ruled out, it is suggested that this might occur due to rapid inactivation of the underlying mechano-sensitive mechanism, which substantially recovers in approximately 2–3 min. This feature fits with the hypothesis that the rapid mechano-sensitive dilatation mediates a feed-forward control of muscle blood flow at the beginning of exercise and may have implications on the way and passive limb movement is employed for the improvement of muscle perfusion.

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