

## ENDOTHELIN -1 AND CARBACHOL: DIFFERENCES IN CONTRACTILE EFFECTS AND MYOSIN PHOSPHORYLATION IN LAMB TRACHEAL SMOOTH MUSCLE

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**Abstract:** Endothelin-1 is a new potent vasoconstrictor peptide produced by the endothelial cells. The contractile effects of endothelin-1 (ET-1) were compared with those of carbachol in lamb tracheal smooth muscle. Equimolar concentrations ( $10^{-6}$  M) of endothelin 1 and carbachol elicit rapidly rising isometric tension which is maintained indefinitely in a steady state when fibres are stimulated with carbachol. Fibre strips exposed to ET-1 cannot maintain peak isometric force beyond 15-20 min and instead these exhibit a decline in tension towards near relaxed state. In addition to an early transient relaxation, ET-1 stimulation results in a 20,000 Da myosin light chain phosphorylation pattern different from that of carbachol stimulation.

**Key words :** endothelin-1                      carbachol                      isometric force  
tracheal smooth muscle                      LC<sub>20</sub> phosphorylation

### INTRODUCTION

Porcine endothelin (ET-1) is a potent vasoconstrictor peptide characterized recently from culture medium of aortic endothelial cells (1). The *in vitro* studies reveal that ET-1 causes vasoconstrictions in almost all arteries and veins of different mammalian species (1-6). The small 21 amino acids peptide with two intrachain disulphide linkages is not only potent hypertensive agent but a strong bronchioconstrictor too (7). ET-1 is an unique vasoactive peptide which elicits most powerful and sustained contractile responses in vascular as well as non-vascular smooth muscles (1, 7).

Intravenous bolus injection of ET-1 into rat results in a triphasic pressure response (1). This begins with a decrease in blood pressure followed by a rapid increase and finally a sustained pressure phase that continues for several minutes. Simultaneously, it has been reported that low doses of ET-1 dilate the isolated perfused rat mesenteries and that this vasodilatory effect is mediated through the release/secretion of

endothelium derived relaxing factor (EDRF) or eicosanoids (8-11).

Phosphorylation of 20,000 Da regulatory myosin light chain (LC<sub>20</sub>) by a calcium and calmodulin dependent myosin light chain kinase is a specific signal that initiates the process of smooth muscle contraction (12). A distinct relationship between contraction and degree of phosphorylation is thus characteristic to excitation contraction coupling in smooth muscle (13-15). Regulation of smooth muscle contraction by regulatory light chain phosphorylation is also supported by correlation between LC<sub>20</sub> dephosphorylation and relaxation (16).

The communication presents observations on the contractile effects of endothelin-1 and another traditional agonist, carbachol in lamb tracheal smooth muscle fibre strips. Since carbachol also produces longer lasting though less potent contractions as compared to endothelin-1, it was sought to know if these contractile effects and corresponding phosphorylation of regulatory myosin light chain also reveal a resemblance between two.

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## METHODS

Fresh lamb tracheae obtained at a slaughter house were brought to the laboratory on ice cold and oxygenated (100%) physiological salt solution (PSS). The physiological salt solution (Ph = 7.50) comprised of (in mM): NaCl (118.00); KCl (5.00); MgCl<sub>2</sub> (1.2); CaCl<sub>2</sub> (1.6); Na<sub>2</sub>HPO<sub>4</sub> (1.2); HEPES buffer (24.00) and glucose (10.00). Smooth muscle layer from tracheae was carefully separated from cartilage including adhering fat and connective tissue. Fibre strips measuring approximately 0.5 mm × 5.00 mm were dissected under binocular microscope and mounted horizontally between a micrometer drive and force displacement transducer (Aksjeselkapet 801, Horten Electronics) for the measurement of isometric force. Mounted fibres which were continuously gassed with 100% oxygen were equilibrated for two hours at 37°C in PSS. During equilibration, optimum fibre length for maximum contractile response (L<sub>0</sub>) was adjusted by partial length tension adjustment using 100 mM KCl in PSS (substitution for NaCl) as a stimulant. Following 20-30 minutes of further equilibration, fibre strips were challenged with appropriate pharmacological agent (ET-1 or carbachol).

Myosin light chain phosphorylation was determined by freezing the fibre strips at desired time point of a contractile event in 25% trichloro-acetic acid solution in acetone precooled to -80°C. Frozen fibre strips were processed for isoelectric focusing and two dimensional gel electrophoresis as per Fischer and Pflitzer (17).

Endothelin-1 (human porcine) was obtained from peptide Institute Inc. Osaka, Japan and carbachol (carbamyl choline chloride) was purchased from Sigma Co.

## RESULTS

Endothelin-1 elicits potent concentration dependent contractions in smooth muscle fibre strips from lamb trachea with a threshold at 10<sup>-11</sup>M. The onset of contractions by ET-1 at lower concentrations (less than 10<sup>-8</sup>M) is slow and requires not less than 20-30 minutes to reach a steady state (Fig. 1A). At higher concentrations, there is an immediate onset and a brisk rise in isometric force lasting 2-5 minutes. The force

rises further till a steady state is reached in about 15 minutes (Fig. 1B). Sometimes spontaneous rhythmic contractions are also observed after an initial burst in isometric tension. A complete contractile event following ET-1 stimulation of fibre strips exhibits two conspicuous features. First, ET-1 induced contraction is preceded by a brief relaxation whose duration is inversely related to the concentration of peptide in organ bath. At higher doses, this transient relaxation is largely overwhelmed by a subsequent rapid contraction. Second, these fibre strips do not maintain peak isometric tension indefinitely. The force either declines rapidly or tend to decline towards resting tension. The relaxed tension is, however, not attained even after as many as 60 minutes of ET-1 stimulation. Only 25% fibre strips could maintain peak tension for 60 minutes following the application of 10<sup>-6</sup> M ET-1 (n=12). At lower ET-1 concentration, this decline although less sharp (Fig 1C) is invariably observed (n=15). This endothelin-1 induced relaxation is equally observed in pig tracheal smooth muscle (n = 4; data not presented).

Equimolar concentrations of carbachol (10<sup>-7</sup> to 10<sup>-6</sup> M), on the other hand, produce a rapidly rising isometric force (Fig 1D) which reaches peak in 2-5 min and is maintained indefinitely (atleast for 60 min in this study; n = 15). Not even a single carbachol induced contraction exhibited any kind of relaxation effect. Differential contractile effects of ET-1 and carbachol become further apparent at the time of steady state tension. Addition of endothelin-1 (10<sup>-8</sup>M) at the time of peak isometric tension following carbachol stimulation, reverses the contractile tension within seconds (Fig. 1E) which touches almost relaxed level in 15-20 minutes and does not recoup thereafter (n = 6). On the other hand, addition of carbachol (10<sup>-7</sup> M to 10<sup>-6</sup>M) to endothelin-1 (10<sup>-8</sup> to 10<sup>-6</sup>M) stimulated fibre strips around 15 min of the contractile event (when fibre strips start exhibiting relaxation) brings isometric force back to steady state level immediately and this peak force is maintained as such indefinitely thereafter (Fig. 2A). These results thus demonstrate that compared to carbachol which elicits longer lasting peak contractile tension, ET-1 possibly exerts a dual function in lamb tracheal smooth muscle. This begins with a brief relaxation followed immediately by contractile phase which finally ends up in a considerable relaxation of fibre strips.

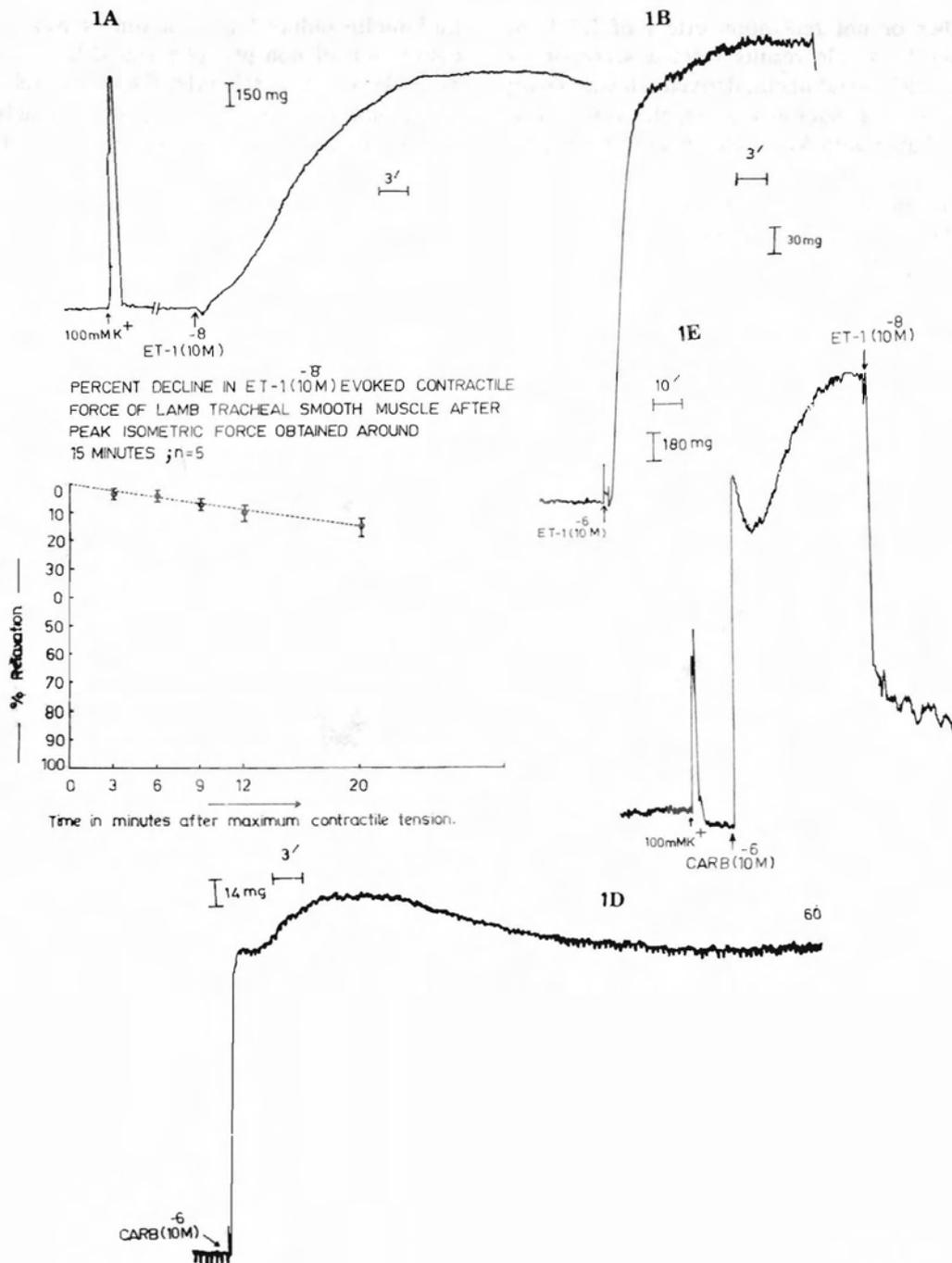


Fig. 1 : Effects of endothelin-1 and carbachol on isometric force and myosin phosphorylation in lamb tracheal smooth muscle. Low doses ( $10^{-8}$  M) elicits (1A) a gradually rising tension compared to a brisk rise (1B) at higher concentration ( $10^{-6}$  M). Transient relaxation at the beginning of contraction at low peptide concentration (1A) and a delayed relaxation after attainment of peak force at all concentrations (1A-C) is characteristically observed. Carbachol, on other hand, propagates a rapidly rising peak tension which is maintained as such indefinitely (1D). This steady state condition is however, reversed by endothelin-1 (1E).

Whether or not relaxation effect of ET-1 on tracheal smooth muscle results from a secretion of substance parallel to endothelin derived relaxing factor (EDRF) in vascular smooth muscle, the fibre strips when preincubated with Methylene blue (10-100  $\mu$ M; n=5) failed to inhibit either the early transient relaxation or ultimate decline in isometric tension (Fig. 2B). Even the potentiation effect on contractile force in such fibre strips is not witnessed.

Endothelin-induced contraction is associated with conversion of non-phosphorylated LC<sub>20</sub> into mono-, di-, and even triphosphorylated forms as well (Fig. 2D). Thus, three to four spots of LC<sub>20</sub> are invariably observed on two dimensional gels when subjected to silver staining. Carbachol stimulation on the other hand, reveals conversion of nonphosphorylated LC<sub>20</sub> into monophosphorylated form only and thus two spots are visible on 2-D gels (Fig. 2C). The characteristic

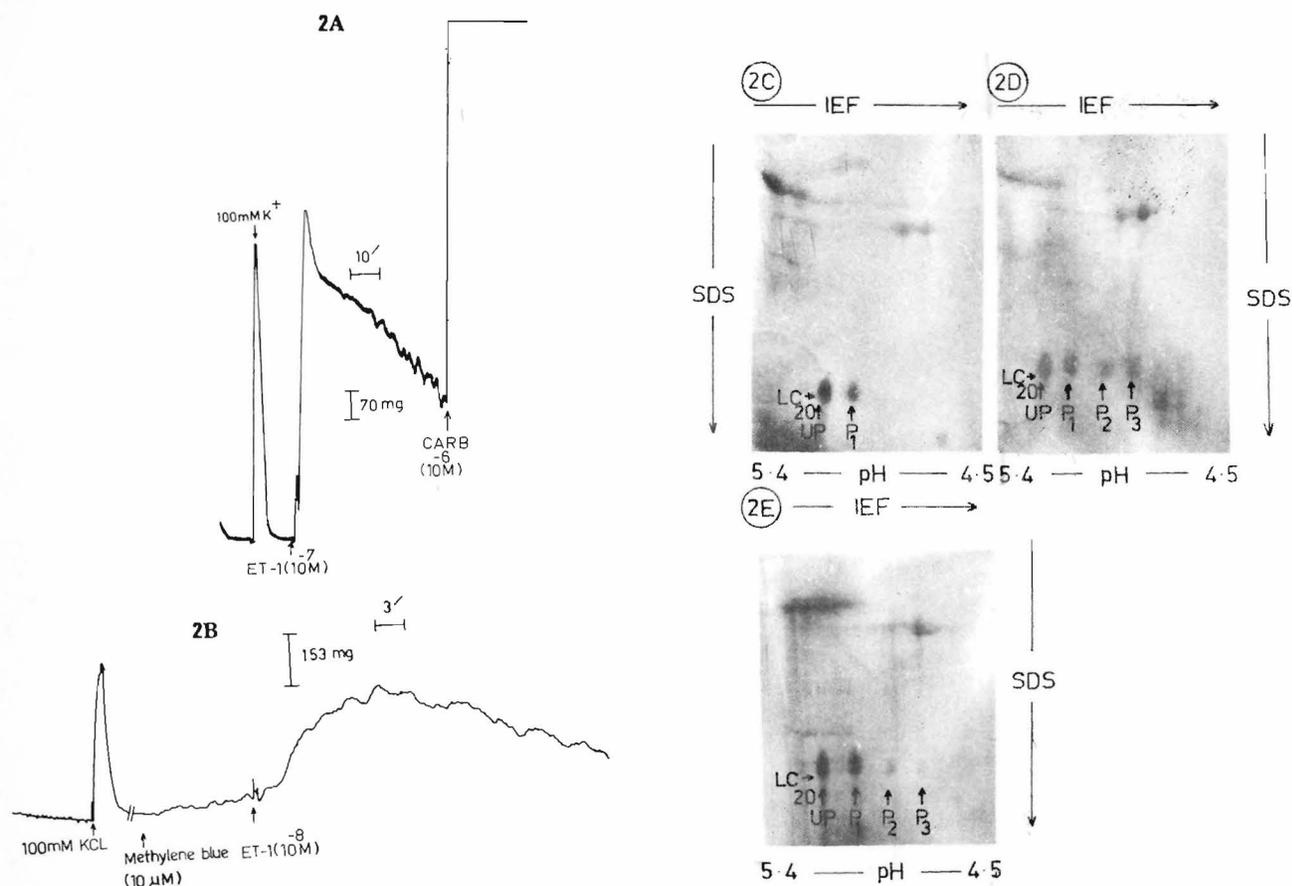


Fig. 2 : Endothelin-1 led decline in isometric force can be brought back to peak steady state level by carbachol addition (2A) and methylene blue inclusion in organ bath has no effect on endothelin induced contractile response of tracheal smooth muscle(2B). Electrophoretic analysis of LC<sub>20</sub> reveals that compared to LC monophosphorylation by carbachol (2C), endothelin-1 converts it into monophosphorylated (P1), diphosphorylated (P2), and triphosphorylated (P3) form (2D & 2E). UP refers to the unphosphorylated LC<sub>20</sub>.

Differences between two agonists become more visible and convincing when phosphorylation pattern of regulatory myosin light chain is analysed.

phosphorylation pattern following endothelin-1 stimulation of the fibre strips is confirmed by the addition of vasoconstrictor peptide to organ bath at the

time of steady state tension following carbachol stimulation. Monophosphorylation pattern characteristic to carbachol stimulation changes into a multiple phosphorylation once again in such fibre strips (Fig 2E).

## DISCUSSION

The present study reveals marked differences between ET-1 and carbachol induced contractile responses in lambtracheal smooth muscle. Endothelin-1 induced contractile response is preceeded by a brief relaxation in contrast to that of carbachol which results in an immediate and brisk rise in isometric force without any relaxation. The two agonists elicit an identical response as far as attainment of peak tension is concerned. However, contractile tensions resulting from the application of two agonists, dissociate from each other during steady state condition. Although more potent than carbachol, ET-1 induced contractile force cannot be maintained indefinitely by fibre strips and instead these exhibit relaxation after some time. On the other hand, carbachol stimulation leads to the propagation of a peak isometric tension that can be maintained in steady state indefinitely. These observations point to some fundamental differences between the mechanisms of action of two agonists.

The immediate but brief relaxation following endothelin-1 application as also reported in porcine coronary artery (1) and in rat mesentery artery (10) has been attributed to the secretion of endothelium derived relaxing factor (8-10) or to eicosanoids (11). There is no structure like endothelium in tracheal smooth muscle but the relaxation of fibres is invariably observed after the attainment of peak isometric force. Further, inclusion of methylene blue, a potential inhibitor of EDRF cannot either potentiate or inhibit the peak contractile response. The brief transient relaxation at the beginning of contractile event as also the late relaxation remain beyond any influence following methylene blue inclusion in bath. This suggests that relaxation of fibre strips especially during late steady state is a feature characteristic to ET-1 induced contractions in lamb tracheal smooth muscle and is not on account of the secretion of a factor equivalent to EDRF reported for vascular smooth muscle (8-11).

Endothelin-1 has been reported to be an unique agonist in producing longer lasting sustained contractions (1,2,7). However, observations summarised in this communication support the contention that ET-1 possibly exerts a dual regulatory function. This begins with a strong contractile effect which ultimately declines to slightly suprabasal level. Maintenance of isometric tension at this level should suffice to account for tone characteristic to smooth muscle as this tension at low level would demand a low cross bridge cycling rate. Such a contention falls in line with the report of Hai & Murphy (18) that tonicity in smooth muscle results following modulations in cross bridge cycling rate. Maintenance of latch state or smooth muscle tone is thus a consequence of low cross bridge cycling rate (18). Endothelin-1 is possibly an *in vivo* peptide which participates in the regulation of smooth muscle tone. That endothelin secretion may possibly contribute to latch state or smooth muscle tone has already been highlighted (2). Further, the reversal of carbachol elicited contractile force by ET-1 and the recoument of ET-1 led declining force by carbachol back to steady state provide experimental support to endothelin-1 in fact modulating the contractile tension in lamb tracheal smooth muscle.

Reversible phosphorylation of 20,000 Da regulatory myosin light chain is a pivotal regulatory event in smooth muscle contraction (12). An analysis of regulatory myosin light chain on two dimensional gel electrophoresis reveals differences in phosphorylation patterns. ET-1 induces a multiple phosphorylation pattern in contrast to monophosphorylation by carbachol. The multiple phosphorylation pattern appears to be a characteristic feature to the maintenance of sustained tension at a slightly suprabasal level or in latch state. On the other hand, monophosphorylation coupled with sustained peak isometric force during steady state condition following carbachol stimulation possibly reflects tension maintenance at high level.

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