Experimental evidence for a non-renin mediated pathway during TAME-esterase induced contractions in rat aorta in vitro

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Possible pharmacological effects of N-α-tosyl L-arginine methyl ester [TAME] were studied on rat aorta strips in vitro. Results showed that [TAME]-esterase was an endothelium dependent component that involved a nitric oxide cyclic-GMP mediated pathway. Furthermore, during activation of Kinin-Kallikrein system, TAME-esterase induced contractions involve degradation of kinins by kininases.

Endothelial cell is a metabolically active component of the vascular wall that can influence vascular tone in many ways. Endothelium is the primary site for the conversion of angiotensin I to angiotensin II (Ref1). It actively metabolizes arachidonic acid to a variety of vasoactive eicosanoid products, most notably prostacyclin, and thromboxane2. Nitric oxide, prostaglandin E2, prostacyclin, endothelium-derived hyperpolarizing factor(s), endoperoxides and the long acting vasoconstrictor peptide endothelin are also liberated from the endothelium in response to several endogenous substances and shear stress3. Inactivation of many circulating vasoactive compounds including bradykinin, adenosine, norepinephrine and prostaglandin E2 tend to take place at endothelium.

Renin activity in the kidney is regulated by the endothelium. Furthermore, angiotensin converting enzyme (ACE) catalyzes the generation of bradykinin as part of the kinin-kallikrein system both of which possess potent vasoactive effects that are either dependent on, or modulated by, endothelium-derived mediators4.

N-α-tosyl L-arginine methyl ester [TAME]-esterase has been demonstrated to be an enzyme, which is involved during the sequence of events leading to the activation of the kinin-kallikrein system5. Furthermore, TAME-esterase induced contractions in toad ileal strips in vitro have been shown to be mediated via a nitric oxide-cyclic GMP pathway6. Results so far thus tend to show that TAME-esterase activity has a significant contribution during contraction of smooth muscles in vitro. Currently there is no experimental evidence regarding action of TAME-esterase on aorta in the literature. Based on our previous work, it has been reported that TAME-esterase is released by activation of Hageman factor, which is an essential component of intrinsic mechanism leading to blood clotting and thus may be a new cardiovascular risk factor among smokers7. The present study was undertaken to further investigate possible pharmacological properties of TAME-esterase on aorta strips in vitro.

Adult male Sprague-Dawley rats weighing between 50-100 g were sacrificed by a severe blow to the head. The thoracic aorta was isolated and mounted in an organ bath containing 25mL of Krebs-Henseleit buffer of the following composition (mM) NaCl 118; KCl 4.7; CaCl2·H2O 2.5, MgSO4·7H2O 1.2; KH2PO4 1.2; NaHCO3 25; Na2EDTA 9.7 mg/L and Glucose (2g/l). To prevent blood clot formation in the dissected aorta, 2 mL of heparin (5,000 IU/L) was added to the buffer in a petri dish. The tissue bath solution was maintained at 37°C in a thermostated water bath. A gas mixture of O2 (95%) and CO2 (5%) was continuously bubbled through the buffer and pH was adjusted to 7.45. Two stainless steel hooks were inserted into the aorta lumen, one was fixed while the other was connected to transducer. Isometric contractile responses were recorded via a force transducer connected to a multipen recorder (Rikadenki Model R50; Japan). Aorta strips were allowed to equilibrate in the medium for 20 min and maintained under an optimal tension of 1.5 g. Presence of intact endothelium was ascertained by acetylcholine (10⁻⁶M)8. No observed
relaxation responses from acetylcholine (4.4x10⁻⁶ M) were taken as an indicator of successful de-endothelialization⁸.

**Effect of N-α-tosyl L-arginine methyl ester (TAME)—**For studying the effect of TAME on aortic strips, a 10⁻¹ M stock solution of TAME was prepared by dissolving 0.38g of TAME (purchased from Sigma, U.K.) in 10 mL of distilled water. Aliquots of this stock solution were used to make serial dilutions ranging from 10⁻¹ to 10⁻¹⁵ M respectively. Twelve aorta strips from 12 rats were used in this series of experiments. Each strip was challenged with 100 µL of TAME, beginning with the lowest dilution (10⁻¹⁵ M). A cumulative dose-response curve was established using the remaining dilutions after stabilization of the strip following any contractile responses or after 3 min in case of no changes occurred. The final concentration of TAME in the bath was 4.4x10⁻⁴ M. In another series of experiments, using same number of aortic strips, endothelium was removed by rubbing the intima surface of aorta with a small glass rod moistened with distilled water. Failure for acetylcholine (4.4x10⁻⁶ M) to induce relaxation was taken as an indicator of successful de-endothelialization⁹.

**Effects of captopril, an ACE Inhibitor—**In addition to TAME, effects of different concentrations of captopril, and an angiotensin converting enzyme (ACE) inhibitor were also studied on seven rat aorta strips. In this series of experiments, aortic strips were pre-incubated with different concentrations of captopril (purchased from Adcock Ingram, Denmark) to give a final concentration of 2.0x10⁻⁵ M, 4.0x10⁻⁴ M and 0.02 M respectively. Following a 20 min incubation period, strips were challenged with different dilutions of TAME. Above experiments in the presence of captopril were repeated on same number of endothelium-denuded aorta strips.

**Effect of a guanylate cyclase inhibitor—**Effects of methylene blue (purchased from Sigma, U.K.), a guanylate cyclase inhibitor was studied on vasoconstriction induced by TAME on aortic strips (n=7). Each strip was pre-incubated with methylene blue (1.0x10⁻³ mM) for 20 min prior to being challenged with different concentrations of TAME. Experiment was repeated using same number of endothelium denuded aorta strips.

**Effects of methylene blue and ACE inhibitor—**Seven aorta strips were pretreated with methylene blue (1.0x10⁻³ mM) for 20 min, followed by another incubation with 1 M captopril (0.02 M) before being challenged with different concentrations of TAME. The experiment was repeated on seven endothelium-denuded aorta strips.

**Effects of L-arginine—**Seven aorta strips were pretreated for 20 min with L-arginine (0.02 mM) before being challenged with different concentrations of TAME. The experiment was repeated on seven endothelium denuded aorta strips.

**Control Experiments—**In each series of experiments, a parallel control strip was included and challenged with 100 µL of distilled water added at 3 min interval.

**Statistical analysis—**All data manipulation and statistical analyses were done using Excel software. Statistical differences between means were assessed by one way analysis of variance (ANOVA). Two way ANOVA was used for analyzing the difference between two concentration-response curves. Once a significant difference was detected, Student’s t test was used to determine the enzyme inhibitor concentration at which significant differences were present.

EC₅₀ values for each pharmacological agent used on the rat aorta strips were determined from respective cumulative concentration curves. Our results showed that TAME induced contraction was significantly inhibited (P<0.05) when rat intact aorta strips were pre-incubated with captopril (1M; Table 1). Table 2 shows mean EC₅₀ values for intact and denuded aortic strips treated with ACE inhibitors (captopril 0.01, 0.2 and 1M), whereas Table 3 shows mean EC₅₀ values for endothelium-intact and endothelium-denuded aortic strips treated with enzyme inhibitor and nitric oxide donor. Mean contractile responses as determined from the cumulative dose response curves were significantly different for endothelium-intact and endothelium-denuded aortic strips challenged with TAME (10⁻¹⁵ to 10⁻¹ M).

There is an evidence, which suggests a renin dependent antihypertensive activity of captopril, but other studies in experimental animals and in humans suggest a non-renin-dependent antihypertensive activity of captopril⁶. Present results demonstrated that in addition to methylene blue, captopril inhibited contractions induced by TAME-esterase in rat aorta strips in vitro. The significant inhibition of TAME-esterase induced contractions could be the result of degradation of kinins by kininase II.
Our present findings have shown that when strips were pre-incubated with captopril (1M), there was a significant inhibition of the contractile response previously seen with TAME alone. However, when the strips were pre-incubated in the presence of methylene blue and captopril simultaneously, TAME-esterase induced contractile responses were more significantly depressed compared to pre-incubation with either one of the pharmacological agents alone. Furthermore, the significant inhibition of contractile responses to TAME-induced contractions further supported the important role of TAME-esterase during activation of the kinin-kallikrein system.

TAME (10^{-15} to 10^{-4} M) elicited a concentration-dependent contraction on both endothelium-intact and endothelium-denuded strips. Mean EC_{50} value calculated for endothelium-intact strips was significantly different from that for denuded strips. It can therefore be concluded that TAME-induced contraction has both endothelium-dependent and endothelium-independent components.

NO synthesized in the endothelium may relax smooth muscle by stimulating guanylate cyclase which synthesizes cGMP. Activation of cGMP-dependent kinase decreases the phosphorylation of yosin light chain, which leads to relaxation. In this study, the concentration of TAME that induced 50% contraction in the presence of methylene blue (2.8x10^{-14} M) in endothelium-intact ring was less as compared to that for intact strips without methylene...
blue $(3.4\times10^{-12} M)$. It can be said that endothelium-dependent component of TAME-induced contraction may depend upon stimulation of guanylate cyclase within smooth muscle\(^6\). Our results showed that TAME induced contractions were significantly inhibited ($p<0.05$) when rat intact aorta strips were pre-incubated with L-arginine but were not significant between intact and denuded aorta strips.

Our results also demonstrated that ACE inhibitors did not significantly depressed TAME-induced contractions in endothelium-intact and endothelium-denuded aortic strips. This data supported the importance of the presence of intact-endothelium for possible effects of ACE inhibitors on TAME-induced contractions in rat aorta strips. This finding has not been previously reported and would tend to confirm our hypothesis that during TAME-esterase induced contractions through activation of kinin-kallikrein system two independent mechanisms are involved. The first mechanism involves an endothelium dependent nitric oxide c-GMP mediated pathway and the second mechanism would be due to the degradation of kinins (bradykinin) by enzyme kininase. Effect of captopril (I M), ACE-inhibitor (Kininase II) on TAME-esterase induced contractions would thus be due to the kininase degradation of bradykinin being blocked.

In conclusion, the present data provided an evidence for a non-renin mediated mechanism of action and present work added on to the experimental evidences about precise role of TAME-esterase in kinin-kallikrein system.

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References