Homocysteine potentiates esterase-induced contraction on rat aorta \textit{in vitro}: A risk factor for atherosclerosis

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Received 24 December 2002; Accepted 5 June 2003

Abstract

The present study was designed to investigate the effect of homocysteine on N-α-L-tosyl arginine methyl ester (TAME) induced contraction of rat thoracic aorta \textit{in vitro}. Aorta was isolated and mounted in an organ bath containing Krebs solution. The effect of TAME was tested on intact aortic strips by challenging with different concentrations of TAME ($10^{-15}$-$10^{-1}$M). The effects of homocysteine were also investigated on TAME-induced contraction on rat aorta strips. Our data showed that the concentration dependent TAME-induced contraction were more prominent when rat aortic strips were pre-incubated with homocysteine.

Key words: homocystine, TAME, endothelial dysfunction, nitric oxide, atherosclerosis

Homosisteinin sıcak aortunda esteraz ile artırılan \textit{in vitro} kasılmayı güçlendirmesi: Atherosclerosis oluşumu için bir risk faktörü

Özet

Bu çalışmada, homosistein etkisi altında N-α-L-tosil arginin metil ester (TAME) ile artırılmış sıcak thoracic aortunda \textit{in vitro} kasılmayı nasıl etkilediği araştırılmıştır. Aort izole edilerek Krebs çözeltisi içeren bir organ banyosuna yerleştirilmiştir. Değişik konsantrasyonlarda ($10^{-15}$-10^{-1}M) TAME çözeltisinde kesik aort stripleri test edilmiştir. TAME etki ettirilmiş bu striplerde ayrıca homosistein etkisi araştırılmıştır. Bulunan sonuçlara göre konsantrasyona bağlı TAME etkili kasılmının önceden homosistein ile inkübe edilen striplerde görülen kasılmaya göre daha göze çarpıcı olduğu saptanmıştır.

Anahtar sözcükler: Homosistein, TAME, endotelyal fonksiyon bozukluğu, nitrik oksit, atherosclerosis

Introduction

Homocysteine is derived from the metabolism of the essential amino acid methionine which is found in greatest concentrations in animal proteins. In humans, dietary animal protein results in a transient rise in plasma homocysteine levels, which peaks at 8 hours and may persist for up to 24 hours (Guttormsen \textit{et al.}, 1994). Homocysteine concentrations are determined by genetic and nutritional factors. Mutations in the genes for enzymes involved in homocysteine metabolism as well as deficiencies of vitamins $B_6$, $B_{12}$ and folic acid, are associated with hyperhomocysteinemia (McCully, 1996).

Evidence indicates that chronic hyperhomocysteinemia is an independent risk factor of atherosclerosis (Stuhlinger \textit{et al.}, 2001). Chronic elevations of plasma homocysteine concentration has
been shown to be associated with stroke, peripheral vascular disease, and myocardial infarction (Boushey et al., 1995). Like hypercholesterolaemia, hyperhomocysteinemia is caused by both genetic and dietary factors and contributes to vascular disease in a large number of patients (Stamper and Malinow, 1995). Elevated homocysteine concentrations are found in almost one-third of all patients with atherosclerosis and levels only 12% above the upper limit of normal (15 μmol/L) are associated with a 3-fold increase in the risk of acute myocardial infarction (Nygaard et al., 1997).

Increasing evidence suggests that the effect of elevated homocysteine are mediated through endothelium dysfunction. In children with cystathionine-β-synthase deficiency and severe hyperhomocysteinemia and in adults with moderate hyperhomocysteinemia, chronically elevated homocysteine concentrations are associated with impaired endothelium-dependent vasodilatation (Tawakol et al., 1997). In primates, elevated homocysteine concentrations following methionine-enriched diet for 4 weeks are associated with vascular endothelium dysfunction (Lentz et al., 1996). Similarly, in normal human subjects, high-dose oral methionine (100mg/kg), which increases plasma homocysteine by 3-to 4-old, is accompanied by a reciprocal fall in brachial artery flow-mediated dilation (Chambers et al., 1998).

It has been shown that homocysteine would reduce the biological activity of nitric oxide (NO) in endothelial cells (Staler et al., 1993). Reduced NO could behave as a trigger mechanism in the development and progression of tissue injury because NO modulates vascular tone, inhibits platelet activation, and attenuates adherence to endothelial (Furchgott and Zadwadski, 1980).

N-α-tosyl L-arginine methyl ester (TAME)-esterase has been demonstrated to be an enzyme, involved in the sequence of events leading to the activation of the kinin-kallikrein system (Subratty & Moonsamny, 1998). Furthermore, it has been reported that TAME-esterase induced contraction in toad ileal strips in vitro was mediated via a NO-cyclic GMP pathway (Subratty & Hossany, 1999).

TAME-esterase has also been described to be a possible new cardiovascular risk factor among smokers (Subratty et al., 2000) and evidence tend to show that TAME-esterase activity has a significant contribution to contraction of smooth muscles in vitro.

We have recently reported that calcium antagonists improve TAME-esterase ‘blunted’ endothelial-dependent relaxation in vitro (Gurib and Subratty, 2001). We have also reported that there is a possible contribution of tyrosine kinases during TAME-esterase induced contractions in aorta (Gurib and Subratty, 2002). It has also been reported that TAME-esterase induced contraction in rat aorta in vitro is mediated through release of prostaglandin(s) as a result of endothelial dysfunction (Gurib and Subratty, 2002).

Based on the previous work, the present study was undertaken to investigate the possible effects of homocysteine on TAME-induced contractions of rat aorta in vitro.

**Materials and Methods**

**Experimental design and surgical procedure**

Adult male Sprague-Dawley rats weighing between 50-100 g were killed by a severe blow to the head. The thoracic aorta was isolated and mounted in an organ bath containing 25 ml of Krebs-Henseleit buffer (mM) NaCl 118; KCl 4.7; CaCl₂·H₂O 2.5; MgSO₄·7H₂O 1.2; NaHCO₃ 25; EDTA 9.7 mg/l and glucose (2 g/l). The pH of the buffer was adjusted to 7.45.

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 95% and 5% CO₂ was continuously bubbled in the buffer. Two stainless steel hooks were inserted into the aorta lumen, one was fixed while the other was connected to transducer. Contractile responses were recorded via an isometric 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 2 g.

**Effects of N-α-tosyl L-arginine methyl ester TAME on rat aorta strips**

For studying the effects of TAME (Sigma, UK) on aortic strips, a 10⁻³ M stock solution of TAME was prepared by dissolving 0.38 g of TAME in 10 ml of distilled water. Aliquots of this stock solution were used to make serial dilutions ranging from 10⁻³ to 10⁻¹ M. 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 concentration (10⁻¹⁵ M).

The procedure was repeated in order of increasing concentration to establish a cumulative dose-response curve after stabilization of the strip following any contractile responses or after 3 minutes in case of no observed changes. The final concentration in the bath was 4 x 10⁻³ M.

**Effect of homocysteine on TAME-induced contraction on rat aortic strips**

In addition to TAME, the effects of homocysteine were also studied on seven rat aorta strips. In this series of experiments, aortic strips were pre-incubated with 500 µl homocysteine (5.0 mmol/l) for 20 minutes before being challenged with 100 µl of TAME, beginning with the lowest concentration (10⁻¹⁵ M).

**Control experiments**

In each series of experiments, a parallel control strip was included from the same aorta. Control aorta strips were challenged with 100 µl of buffer solution added at 3-minute intervals during the experiments investigating the effect of TAME. For test strips 100 ml of different dilutions of TAME were added at increasing concentrations and the contractile responses, if any, were recorded as described above.

**Data analysis**

All results are expressed as mean ± SE. Manipulation and statistical analyses of the data 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 homocysteine concentration at which significant differences were present. P values less than 0.05 were considered as statistically significant.

**Results**

The results of the present study demonstrated that TAME had a dose-dependent effect causing contractions of aorta strips *in vitro* (Figure 1A). There was no effect by the application of buffer solution alone (Figure 1C). Contractile responses obtained with TAME following preincubation of strips homocysteine were presented in Figure 1B. The same set of data was expressed as a percentage of maximal response in

**Figure 2** Effects of homocysteine on TAME-induced contractions on rat aorta *in vitro*. 

### *EC₅₀* (effective concentration of pharmacological agent producing half the maximal response) was calculated by linear interpolation for
each pharmacological agent applied on the rat aorta strips, using respective cumulative concentration curves (Table 1). The results showed that TAME-esterase induced contractions were amplified (p>0.05), when rat intact aorta strips were pre-incubated with homocysteine.

### Discussion

Hyperhomocysteinemia has long been recognized as an independent risk factor for the pathogenesis of arteriosclerosis and venous thrombosis (Schlaich et al., 2000). Hyperhomocysteinemia, characterized by accelerated atherosclerosis, is believed to induce endothelial cell injury and promote atherothrombosis by supporting the generation of hydrogen peroxide (Upchurch et al., 1997). Despite the clinical significance of homocysteine, however, the molecular mechanisms of homocysteine-induced arteriosclerosis have not been completely elucidated (Lee & Wang, 1999). It is well established that endothelial dysfunction, which is characterized by loss of control in NO, production occurs in a variety of cardiovascular disorders. A decrease in level of NO leads to a cascade of pathophysiologic events resulting in neutrophil infiltration into inflamed tissue a process which is regulated by adhesion molecules (Lefer and Lefer, 1996).

Stuhlinger et al. (2001) have reported that when endothelial or nonvascular cells were exposed to DL-homocysteine or to its precursor L-methionine, dimethylarginine (ADMA) concentration in the cell culture medium increased in a dose- and time-dependent fashion. This effect was associated with the reduced activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA. Furthermore, homocysteine-induced accumulation of ADMA was associated with reduced NO synthesis in endothelial cells and segments of pig aorta. The antioxidant pyrrolidine dithiocarbamate preserved DDAH activity and reduced ADMA accumulation. Moreover, homocysteine reduced the activity of recombinant human DDAH in a cell free system in a dose-dependent manner by direct interaction between homocysteine and DDAH. It was concluded that homocysteine post-translationally inhibits enzymatic activity of newly synthesized and causing ADMA to accumulate and inhibit nitric oxide synthesis.

The findings from the present study showed that homocysteine amplified the effect on TAME-esterase induced contractile response on rat aorta. One plausible mechanism for these observed effects could be due to homocysteine causing endothelial dysfunction by down regulating the synthesis of NO. From the literature it is evident that homocysteinemia inhibits the important role of NO in preventing endothelial dysfunction (Schlaich et al., 2000). This is probably due to the reduced endothelial basal NO release in the arterial aviculture, and may explain the known effect of homocysteine to impair endothelial-mediated NO-dependent vasodilatation.

In conclusion, our study further demonstrates the potential role of TAME in the cascade of events leading to endothelial dysfunction. However further work is needed to establish a relationship between the cardiovascular effects of NO, plasma homocysteine levels and cardiovascular diseases, our data are complementary to the more traditional NO-induced stimulation of guanylate cycles.

### References


Gurib FBH and Subratty AH. Involvement of Kinin Kallikrein and Prostaglandin in TAME-esterase induced

### Table 1: Mean EC\textsubscript{50} values for endothelium-intact aorta strips treated with homocysteine. In statistical evaluation of EC\textsubscript{50} data, each of two contraction data was against its own control data.

<table>
<thead>
<tr>
<th>Chemical agents</th>
<th>No. of strips</th>
<th>Mean EC\textsubscript{50} values (M)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAME (10\textsuperscript{-15} – 10\textsuperscript{-1} M)</td>
<td>12</td>
<td>4.0 x 10\textsuperscript{-14}</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>TAME (10\textsuperscript{-15} – 10\textsuperscript{-1} M) pre-incubated with Homocysteine (5.0 mmol/l)</td>
<td>7</td>
<td>2.8 x 10\textsuperscript{-14}</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
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