Modulation of vascular tone by endothelin-1: role of preload, endothelial integrity and concentration of endothelin-1

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1 Endothelin-1 (ET-1) has been shown to exert both arterial relaxant and constrictor effects. To examine the mechanisms of these divergent effects, rat aortic rings were suspended in an organ bath (baseline preload, 5 g) and exposed to ET-1 (10^{-11} to 10^{-7} M). ET-1 contracted these rings in a concentration-dependent fashion.

2 When aortic rings were contracted with noradrenaline (NA) to 1 g of tension, ET-1 caused further contraction of these rings. In rings precontracted to 2 to 4 g of tension, low concentrations of ET-1 (10^{-11} to 10^{-9} M) caused a significant relaxation, but high concentrations ($\geq 5 \times 10^{-9}$ M) caused a marked contraction, indicating both relaxant and contractile effects of ET-1 depending on the preload and ET-1 concentration.

3 To determine the mechanism of ET-1-induced relaxation, aortic rings were pretreated with the cyclo-oxygenase inhibitor indomethacin, N^G -monomethyl-L-arginine (L-NMMA) an inhibitor of synthesis of endothelium-derived relaxing factor (EDRF), or oxyhaemoglobin (Hb) which decreases the activity of EDRF, prior to their exposure to ET-1. Both indomethacin and L-NMMA markedly ($P < 0.01$) attenuated ET-1-induced relaxation, whereas Hb totally abolished it. Removal of the endothelium from aortic rings also abolished ET-1-mediated relaxation.

4 The relaxant effect of ET-1 in NA-precontracted rings was associated with marked accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP), whereas ET-1-induced contraction of quiescent rings was not.

5 In manually stretched rings (4 g of tension), ET-1 caused only concentration-dependent contraction, but no cyclic GMP accumulation.

6 Thus, ET-1 contracts rat aortic rings with intact endothelium and those which are passively stretched. However, stimulation of rat aortic rings with NA to modest tension alters the contractile effect of ET-1 to a potent relaxant effect. The ET-1-mediated relaxation in this setting appears to be endothelium-dependent and is related to release of both cyclo-oxygenase products and EDRF.

Keywords: Endothelium; endothelium-derived relaxing factor; endothelin-1; oxyhaemoglobin

Introduction

Endothelin-1 (ET-1), characterized as a 21-amino acid peptide released from cultured porcine endothelial cells (Yanagisawa *et al.*, 1988) exerts potent vasoconstrictor effect in a variety of mammalian blood vessels. Its levels in plasma are increased in myocardial ischaemia and renal insufficiency (Miyachi *et al.*, 1989; Salminen *et al.*, 1989; Tomita *et al.*, 1989), and it has been suggested that it may exert a vasoconstrictor influence in these disease states (Salminen *et al.*, 1989; Stewart *et al.*, 1991). There is also evidence that ET-1 exerts a vasorelaxant effect, especially when the intravascular pressure is high or when the concentrations of ET-1 are large (de Nucci *et al.*, 1988; Bradley *et al.*, 1990; Perreault & de Marte, 1991).

ET-1 has been shown to induce release of endothelium-derived relaxing factor (EDRF) in intact blood vessels (Lipp-ton *et al.*, 1988; Warner *et al.*, 1989). EDRF relaxes vascular smooth muscle via stimulation of guanylate cyclase (Furchgott & Zawadski, 1980). Similar release of EDRF from blood vessels has been reported in response to several other vascular smooth muscle agonists, and the vasoconstrictor effects of these agonists are enhanced in the absence of endothelium (Cocks & Angus, 1983; Griffith *et al.*, 1984; Sakuma *et al.*, 1987). The release of EDRF in response to these agonists becomes apparent when the tension in the

vascular beds is relatively high (Lawson *et al.*, 1989). ET-1 has also been shown to release eicosanoids from rabbit isolated perfused kidney and spleen (Rae *et al.*, 1989).

We have examined the effects of ET-1 on rat aortic contraction. Since the overall effects of agonists may be dependent on preload as suggested by de Nucci *et al.* (1988), Bradley *et al.* (1990) and Lawson *et al.* (1989), we studied the effects of ET-1 on rat aortic rings exposed to a wide range of preloads.

Methods

Preparation of rat aortic rings

Sprague-Dawley rats (average body weight 225 g) were anaesthetized with halothane and killed. The thoracic aortae were quickly removed and placed into aerated (95% O₂, 5% CO₂) Krebs-Ringer buffer (composition in mM: NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, NaHCO₃ 12.5, Na-EDTA 0.01 and dextrose 11.1 pH 7.4). Excess adventitious tissue was carefully removed and the vessels were cut into 3–4 mm rings. The vessels were washed with buffer, mounted on wire stirrups, suspended in tissue baths filled with buffer at 37°C and connected to force transducers (Grass Instruments, Quincey, MA) to record changes in isometric force (Lawson *et al.*, 1989).

The rings were stretched to a preload of 5 g and allowed to equilibrate for approximately 2 h. This preload was observed to be appropriate to determine dose-response curves to a

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variety of agonists, including noradrenaline (NA). During the period of equilibration, the buffer was changed every 15 min, and 95% O₂ and 5% CO₂ continuously bubbled. Following equilibration, the vascular rings were tested for viability by addition of 20 mM KCl. Endothelial integrity was determined in these rings by the presence of characteristic 60 to 80% relaxation in response to acetylcholine (ACh, 10⁻⁶ M). In some rings, endothelium was removed by gently passing a cotton-tipped swab through the lumen; these rings failed to relax in response to ACh (Lawson *et al.*, 1989), but relaxed promptly in response to nitroglycerin (10⁻⁶ M) indicating intact vascular smooth muscle function.

Experimental protocol

Aortic rings were exposed to gradually increasing concentrations of ET-1 (10⁻¹¹ to 10⁻⁷ M). Following stable contraction, rings were exposed to ACh (10⁻⁶ M). Parallel sets of aortic rings were precontracted with NA (10⁻¹⁰ to 10⁻⁷ M) to achieve varying degrees of tension (1, 2 and 4 g). These NA-precontracted rings were then exposed to ET-1 (10⁻¹¹ to 10⁻⁷ M). Following sustained effects of ET-1, ACh was added to the organ bath to determine the presence or absence of vascular endothelium.

In parallel experiments, aortic rings were treated with the cyclo-oxygenase inhibitor indomethacin (10⁻⁵ M) or the competitive inhibitor of EDRF synthesis, N^G-monomethyl L-arginine (L-NMMA, 5 × 10⁻⁵ M) (Dinerman *et al.*, 1991). Other aortic rings were treated with oxyhaemoglobin (10⁻⁵ M), which binds EDRF (Martin *et al.*, 1985). These concentrations of inhibitors were selected based on previous studies (Dinerman *et al.*, 1991), which showed that 10⁻⁵ M indomethacin decreases prostaglandin synthesis by about 80%, 5 × 10⁻⁵ M L-NMMA decreases ACh-mediated relaxation by about 75%, and 10⁻⁵ M oxyhaemoglobin abolishes the ACh-mediated relaxation. In all these rings, 3 to 4 g of tension was achieved with NA. Following sustained contraction, these rings were exposed to ET-1. Other aortic rings, from which the endothelium had been removed, were also contracted with NA to achieve approximately 3–4 g of force and then exposed to ET-1.

To examine if the effects of ET-1 on NA-precontracted rings were due to increased tension, other aortic rings were stretched manually to a tension of 4 g. After the tension was stable, the rings were exposed to ET-1.

Measurement of cyclic GMP

Manually stretched, NA or ET-1-contracted rings, and other rings exposed to low ET-1 concentrations (5 × 10⁻⁹ M) following contraction with NA or manual stretch were flash-frozen in 1 ml of 0.5 mM isobutylmethylxanthine (IBMX) and 10% trichloroacetic acid and stored at -70°C. These rings were homogenized and sonicated. After centrifugation (2500 g for 15 min at 4°C), the resulting supernatants were extracted 4 times in water-saturated ethyl ether (volume 5 ml) and dried under a stream of air. Acetylated guanosine 3':5'-cyclic monophosphate (cyclic GMP) was measured by radioimmunoassay with an ¹²⁵I-kit (New England Nuclear, Boston, MA, U.S.A.). The minimum detectable limit of cyclic GMP assay was 2.5 fmol/0.1 ml. The extraction efficiency using [³H]-cyclic GMP was over 95%. All measurements were made in duplicate (variation < 10%). The cyclic GMP levels were expressed in fmol mg⁻¹ tissue.

Reagents

L-NMMA was a gift of Dr S. Moncada, Beckenham, UK. Indomethacin was a gift from Merck, Sharp and Dohme, West Point, PA, U.S.A. ET-1 was purchased from Peninsula Laboratories, Belmont, CA, U.S.A. Oxyhaemoglobin was prepared as described previously (Dinerman *et al.*, 1991). All

other supplies were purchased from Sigma Chemical, St. Louis, MO, U.S.A.

Statistical analysis

Aortic ring tension was divided by the wet weight of the ring to obtain the isometric tension in g mg⁻¹ tissue. All data are expressed as mean ± s.e. and are based on a minimum of four experiments from four separate rats. Statistical analysis was performed with Student's *t* test for paired and unpaired observations, as appropriate. A *P* value of < 0.05 was considered significant. The effects of various interventions on aortic ring contraction or relaxation are expressed as percentage change from baseline contraction.

Results

Effects of endothelin-1 on rat aortic ring contraction

ET-1 induced a slow, concentration-dependent contraction of all aortic rings. The contraction was greater in rings without endothelium than in those with intact endothelium. The increased sensitivity of rings without endothelium to ET-1 is in keeping with previous observations (Miller *et al.*, 1989; Folger *et al.*, 1991), which indicated increased contraction in response to a variety of agonists, including ET-1, in the absence of intact endothelium.

Endothelin-1 and noradrenaline-precontracted rings

The effects of ET-1 varied markedly when the tension of the rat aortic rings was changed with NA (Figures 1 and 2). When the rings were contracted with NA to approximately 1 g of tension, ET-1 caused an additional contraction. The overall contraction was equal to the sum of the contractile actions of NA and ET-1. When the tension with NA was maintained at approximately 2 g, low concentrations of ET-1 evoked gradual relaxation, but higher concentrations caused contraction of the aortic rings. When the aortic rings were precontracted with NA to approximately 3 to 4 g tension, very low concentrations of ET-1 (10⁻¹¹ to 10⁻⁹ M) caused a potent and rapid relaxation; high concentrations (≥ 5 × 10⁻⁹ M), however, evoked a potent contraction. All these vascular rings relaxed in response to ACh.

In parallel experiments, aortic rings contracted with NA to different tensions were observed for 20 min. However, no spontaneous relaxation or contraction was observed, indicating that the effects of ET-1 were indeed caused by ET-1 and were not spontaneous occurrences.

Endothelin-1 and manual stretch

When aortic rings were manually stretched to 1, 2 or 4 g of tension (above the baseline preload of 4 g), ET-1 caused a concentration-dependent contraction (Table 1). This was in marked contrast to NA-precontracted rings which relaxed in response to low concentrations of ET-1. In these rings, ACh also failed to cause any relaxation.

Mechanism of endothelin-1 mediated rat aortic ring relaxation

To determine the mechanism of ET-1-mediated vascular relaxation, parallel sets of rat aortic rings were contracted with NA to a preload of 3–4 g and then exposed to ET-1, while other rings were treated with indomethacin, L-NMMA or oxyhaemoglobin, or endothelium was removed from the rings. Similar tension was achieved in all rings by adding different amounts of NA (Figure 3).

Both L-NMMA and oxyhaemoglobin caused contraction of NA-contracted rings (Figure 3), presumably because both these agents inhibit the vasorelaxant effect of EDRF. Indo-

methacin slightly decreased the rat aortic ring contraction in response to NA. However, the peak tension in the rings prior to addition of ET-1 was similar in all rings.

Indomethacin significantly decreased the vasorelaxant effect of ET-1, but did not abolish it. L-NMMA also markedly decreased the vasorelaxant effect of ET-1, but like indomethacin did not abolish it. Treatment of vascular rings with oxyhaemoglobin on the other hand totally abolished the vasorelaxant effects of ET-1 (Figure 3, Table 2). The relaxant effect of ACh was present in the indomethacin-treated rings, attenuated in the L-NMMA-treated rings, and absent in the oxyhaemoglobin-treated rings (Figure 3). In the NA-precontracted rings, without endothelium, ET-1 failed to cause relaxation at any concentration (Figure 3, Table 2).

Cyclic GMP accumulation

Levels of cyclic GMP increased markedly in NA-precontracted rings from the quiescent state, and further increased

Figure 1 Effect of endothelin-1 (ET-1) on rat aortic rings with intact endothelium contracted to different preloads with noradrenaline (NA). With no prior exposure to NA, ET-1 caused slow but sustained contraction. When another aortic ring from the same aorta was contracted to 1 g preload, ET-1 caused additional concentration-dependent contraction. In other aortic rings precontracted to higher preloads (2 to 4 g), low concentrations of ET-1 caused relaxation, whereas higher concentrations induced contraction. The relaxation is very rapid as the preload is increased (bottom panel).

Figure 2 Dual vasoconstrictor and relaxant effects of endothelin-1 (ET-1) on rat aortic rings exposed to various preloads. Data from 4 to 8 experiments are presented as mean with s.e. shown by vertical bars.

upon their exposure to ACh (10^{-6} M) indicating EDRF release. ET-1 alone (10^{-8} M) which caused marked contraction of aortic rings had no effect on accumulation of cyclic GMP. However, treatment of NA-precontracted rings with ET-1 (5×10^{-9} M) resulted in a marked increase in cyclic GMP content ($P < 0.02$ vs ET-1 alone or NA alone-precontracted rings), indicating that ET-1 caused EDRF release in NA-treated rings. This increase in ET-1 was minimal in rings that were exposed to ET-1 after manual increase in tension (Table 3).

Discussion

The observations of variable effects of ET-1 on rat aortic rings precontracted to different tensions with NA *in vitro* are novel. At low tension, ET-1 caused an additional contraction of rat aortic rings and the overall effect seemed to represent cumulative interaction between NA and ET-1. Tabuchi *et al.* (1989) also demonstrated potentiation of the vasoconstrictor effects of catecholamines by ET-1. At higher NA-induced tensions, low concentrations of ET-1 ($\leq 5 \times 10^{-9}$ M) relaxed the vascular rings, whereas high concentrations ($\geq 10^{-8}$ M) induced marked contraction. These dual effects were particularly striking in rings exposed to a tension of 3 to 4 g above the baseline preload when the lowest concentration of ET-1 induced a consistent and prompt relaxation (Figure 2). In contrast to the relaxant effect of ET-1 in NA-precontracted rings is the total absence of ET-1-mediated relaxation in manually stretched rings (Table 1). These observations imply

Table 1 Effect of endothelin-1 (ET-1) on manually stretched rings

Manual stretch (g)	Tension (g mg ⁻¹)				ACh (-log M) 6
	11	ET-1 (-log M) 10	9	8	
1	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	1.3 ± 0.3	1.3 ± 0.4
2	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	1.5 ± 0.3	1.4 ± 0.3
4	1.4 ± 0.2	1.5 ± 0.3	1.5 ± 0.3	2.0 ± 0.3	1.9 ± 0.3

Data from 4 experiments as mean ± s.e. Manually stretched rings were exposed to cumulative doses of ET-1 followed by a single dose of acetylcholine. ET-1 did not cause relaxation in any of the rings.

Figure 3 Representative experiments showing mechanism of endothelin-1 (ET-1)-mediated relaxation. Both indomethacin (Indo) and the EDRF synthesis inhibitor N^G -monomethyl-L-arginine (L-NMMA) attenuated the relaxant effect of ET-1, whereas oxyhaemoglobin (Hb) and removal of endothelium (Endo-) abolished the vasorelaxation.

that vascular stretch caused by pharmacological means alters the vascular response to ET-1 in a fashion that is not observed in manually stretched rings.

With respect to the mechanism of ET-1-induced relaxation of NA-precontracted rat aortic rings, it appears that both cyclo-oxygenase products and EDRF are important mediators, since both indomethacin and L-NMMA (competitive inhibitor of EDRF synthesis) decreased the ET-1-mediated relaxation. In contrast to inhibition of relaxation by indomethacin and L-NMMA, oxyhaemoglobin, which binds to EDRF (Martin *et al.*, 1985; Dinerman *et al.*, 1991) and stimulates synthesis of vascular smooth muscle constrictor

Table 3 Guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation in rat aortic rings

	Cyclic GMP (fmol mg ⁻¹)
Quiescent tissues	39 ± 10
NA-precontracted rings	69 ± 15†
NA + ACh	301 ± 71¶
ET-1 (10 ⁻⁸ M)-contracted rings	52 ± 5
NA + ET-1 (10 ⁻⁸ M)	137 ± 43*
Manual stretch + ET-1 (10 ⁻⁸ M)	78 ± 40

Data from 4 experiments as mean ± s.e. Noradrenaline (NA) was used in a concentration required to achieve 4 g of tension. In other experiments, rings were manually stretched to 4 g of tension. ACh-acetylcholine.

† $P < 0.02$ vs quiescent rings.

¶ $P < 0.02$ vs control NA-precontracted rings.

* $P < 0.02$ vs NA-or endothelin-1 (ET-1) precontracted or manually stretched rings.

prostaglandins (Toda, 1990), abolished ET-1-mediated relaxation. Particularly noteworthy is the observation that ET-1 failed to cause any relaxation of de-endothelialised rings, indicating that ET-1 induced relaxation is dependent on intact endothelium.

The importance of EDRF release became apparent from cyclic GMP measurements which increased in NA-precontracted rings exposed to ET-1. A similar increase in cyclic GMP in response to ET-1 in rat aortic segments with intact endothelium has recently been shown by Topouzis *et al.* (1991). Importantly, NA induced contraction was associated with almost a doubling of cyclic GMP accumulation. It may be proposed that ET-1 at low concentrations augments NA-mediated cyclic GMP accumulation, whereas high concentrations of ET-1 overcome the release of EDRF.

Previous studies in a variety of blood vessels from different animals (Cocks & Angus, 1983; Griffith *et al.*, 1984; Sakuma *et al.*, 1987; Lawson *et al.*, 1989) show that EDRF is released from endothelium in response to high concentrations of agonists. This release of EDRF may not be antagonized by specific receptor blockers (Lawson *et al.*, 1989), suggesting that EDRF is released from endothelium when vascular tension increases in response to a pharmacological stimulus. Increase in blood flow in human coronary arteries with intact and functional endothelium is also associated with release of EDRF; and this release of EDRF is diminished when endothelium is dysfunctional (Nabel *et al.*, 1990). The present observations of ET-1-mediated aortic ring relaxation when the tension was increased pharmacologically and the inhibition of relaxation by L-NMMA or oxyhaemoglobin are in accordance with this concept.

Rae *et al.* (1989) documented release of eicosanoids,

Table 2 Effect of indomethacin, N^G -monomethyl-L-arginine (L-NMMA), oxyhaemoglobin and removal of endothelium on endothelin-1 (ET-1)-mediated rat aortic ring relaxation

Treatment	0	11	Tension (g mg ⁻¹)			
			Endothelin-1 (10)	Endothelin-1 (9)	Endothelin-1 (8.3)	Endothelin-1 (8)
Buffer alone (n = 8)	1.24 ± 0.04	1.21 ± 0.03	1.03 ± 0.04	0.73 ± 0.06	0.93 ± 0.12	1.23 ± 0.10
Indomethacin (n = 4)	1.21 ± 0.05	1.21 ± 0.05	1.20 ± 0.06†	1.10 ± 0.10†	1.20 ± 0.10*	1.22 ± 0.10
L-NMMA (n = 4)	1.23 ± 0.05	1.23 ± 0.06	1.21 ± 0.08†	1.05 ± 0.07†	1.20 ± 0.08*	1.24 ± 0.07
Oxyhaemoglobin (n = 4)	1.22 ± 0.04	1.24 ± 0.06	1.27 ± 0.10†	1.29 ± 0.08†	1.31 ± 0.06*	1.32 ± 0.07
Removal of endothelium (n = 4)	1.23 ± 0.06	1.23 ± 0.07	1.28 ± 0.11†	1.30 ± 0.06†	1.33 ± 0.07*	1.38 ± 0.08

Data are expressed as mean ± s.e.

Tension before endothelin-1 was same in all rings.

† $P < 0.01$ and * $P < 0.05$, both as compared to endothelin-1-mediated relaxation in buffer alone-treated vascular rings.

mainly prostaglandin E_2 (PGE_2) and to a lesser extent PGI_2 and thromboxane A_2 (TxA_2) from perfused kidney and spleen of rabbits in response to ET-1. Mehta *et al.* (1984) showed that administration of TxA_2 -cyclic endoperoxide analog U46,619 in intact dogs is associated with release of eicosanoids, PGI_2 and TxA_2 . These observations of eicosanoid release in response to different and unrelated vasoconstrictors suggest that cyclo-oxygenase products are released from endothelium in an attempt to autoregulate vascular tone, when intraluminal stress increases. Our observation of blunting of vasorelaxant effect of low concentrations of ET-1 by indomethacin may be a manifestation of NA-induced release of arachidonate metabolites. Some other investigators, however, have not identified attenuation of the vaso-depressor effects of ET-1 in rats, cats or piglets by indomethacin (Bradley *et al.*, 1990; de Nucci *et al.*, 1988; Hasunuma *et al.*, 1990; Lipton *et al.*, 1989; Perreault & De Marte, 1991). The precise reasons for this disparity are not known, but the *in vitro* nature of the present studies (vs. the *in vivo* studies by others) may relate to these differences.

The importance of the dual actions of ET-1 depending on its concentration is exemplified by some recent studies. de Nucci *et al.* (1988) observed ET-1-mediated rise in arterial pressure in rats with low blood pressure (low preload) and a decrease in arterial pressure in rats with high blood pressure (high preload). In studies in newborn piglets (Bradley *et al.*, 1990), low doses of ET-1 increased coronary blood flow, whereas high doses of ET-1 had the opposite effect. Perreault & De Marte (1991) have recently shown ET-1-induced concentration-dependent contraction of pulmonary vasculature of the newborn pig where the baseline pressure was low; however, in accordance with our studies they observed relaxation of pulmonary vasculature by low concentrations of ET-1 when the baseline pressure was high.

An important aspect of the present study relates to the effect of ET-1 on manually stretched rings. These rings were stretched to the same tension as the pharmacologically-treated rings which demonstrated relaxation and cyclic GMP accumulation in response to low concentrations of ET-1. ET-1 caused a concentration-dependent contraction in these

rings without any evidence for relaxation or cyclic GMP accumulation. ACh also did not relax the manually stretched rings. These observations suggest that NA treatment results in active tension of the vessel wall and primes or modulates the endothelium in a fashion that ET-1 and ACh become inducers of EDRF release. Vascular ring contraction in response to the thromboxane A_2 receptor agonist, U46,619, has also been associated with EDRF release from rat aortic rings with intact endothelium, and the contractile response to this agonist is enhanced in the presence of EDRF inhibitors or when the endothelium has been removed (Dinerman & Mehta, 1990; Folger *et al.*, 1991). It is possible that low concentrations of ET-1 potentiate EDRF release by NA in rat aortic rings and other vascular tissues with intact endothelium. On the other hand, manual fixed stretch of the rat aortic rings will not permit their tension to decrease in response to ET-1 or ACh. However, when the manually stretched rings are further contracted with NA to an additional 2–3 g of tension, both ET-1 and ACh promptly induce vasodilatation (data not shown). In this regard, high vascular tension in the *in vivo* settings represents pharmacological tension in the tissues, and ET-1 promptly decreases arterial tone. However, high concentrations of ET-1 will consistently override the effect of EDRF and cause contraction of the vascular tissues both *in vitro* and *in vivo*.

In summary, this study shows concentration-dependent arterial relaxant and constrictor effects of ET-1. This substance may serve to regulate vascular tone by inducing release of EDRF and cyclo-oxygenase products in physiological states, but exert potent vasoconstrictor influence on the vascular smooth muscle when endothelial function is not intact. In conditions of endothelial dysfunction, such as acute myocardial ischaemia and renal insufficiency, high circulating levels of ET-1 (Miyauchi *et al.*, 1989; Salminen *et al.*, 1989; Tomita *et al.*, 1989; Stewart *et al.*, 1991) may have a role in the genesis and perpetuation of tissue ischaemia.

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