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Thiorphan enhances bradykinin-induced vascular relaxation in hypoxic/hyperkalaemic porcine coronary artery

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Abstract

Relaxation induced by bradykinin is diminished by hypoxia in epicardial coronary arteries. The bradykinin-degrading enzyme, neutral endopeptidase (NEP, EC.3.4.24.11), is a potential target for coronary artery vasodilators. In this study, we examined the effect of thiorphan, an inhibitor of NEP, on the tone of porcine isolated coronary artery under hypoxic conditions. Endothelium-intact porcine isolated coronary artery rings were isometrically contracted with a prostaglandin $F_{2\alpha}$ analogue (U46619, 0.75 μM) and potassium chloride (KCl, 30 mM), and relaxed with bradykinin (1–1000 nM) under normoxic (partial pressure of oxygen, $pO_2 \sim$ 90–100 mmHg) and moderately hypoxic ($pO_2 \sim$ 50–60 mmHg) conditions. Experiments were performed to study the effects of 30 min pre-treatment with the NEP-inhibitor, thiorphan (10 μM), both at physiological and at low pO_2s. Hypoxia inhibited the bradykinin-induced relaxation in porcine epicardial coronary arteries. In normoxia, thiorphan significantly enhanced the decrease of coronary tone produced by bradykinin (1–10 nM) when U46619 was used as contractile agent. Under hypoxic conditions, in U46619 contracture, thiorphan did not influence, but in KCl contracture it enhanced the magnitude of relaxations induced by bradykinin. In the absence of bradykinin, thiorphan had no significant effect on the basal, KCl- and U46619-elevated tones and on the hypoxia-induced decrease of coronary artery tone. Inhibition of NEP-enzyme activity may effectively improve the relaxing capacity of epicardial coronary arteries under hypoxic/hyperkalemic conditions. This effect could be potentially utilized when the endothelial function and relaxation of the coronary arteries are impaired under clinical conditions.

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Introduction

The presence of the intact vascular endothelium is of crucial importance in the regulation of the coronary circulation. Perioperative handling and surgical procedures of the heart have been suggested to depress the vascular endothelial function by mechanical, physical and chemical ways (He & Yang 1996; Saitoh et al 1997). There is evidence that endogenous bradykinin is involved in the vasomotor control of conduit-type coronary arteries and appears to have cardioprotective actions (Drexler & Hornig 1999; Blais et al 2000). Bradykinin is an endothelium-dependent vasodilator and its effect is mediated by nitric oxide; endothelium-dependent hyperpolarizing factor is involved in the injury of the coronary circulation (He 1999; Krassói et al 2000). For potentiating the beneficial effects of bradykinin, one strategy is to elevate the bradykinin concentration by inhibition of kinin-degrading enzymes. In addition to the angiotensin-converting enzyme (ACE), the presence of neutral endopeptidase enzyme (NEP, neprilysin, enkephalinase, EC.3.4.24.11) has also been demonstrated in human hearts subjected to cardiac transplantation (Blais et al 2000).

Hypoxia is a common risk factor during heart surgery, which depresses the energetic balance of the heart muscle and suppresses the blood supply to the heart by decreasing endothelium-dependent relaxation (Hashimoto et al 1993). It has been shown that bradykinin-induced coronary relaxation is diminished by hypoxia, thereby an increased vasomotor tone of the epicardial coronary arteries may further decrease the oxygen supply to the heart muscle (Vedernikov et al 1991). The relevance of pharmacologic interaction between bradykinin and NEP-inhibitors under hypoxic conditions has recently been supported in diseased human hearts. A significant role

of NEP in the metabolism of bradykinin was demonstrated in ischaemic and in idiopathic dilated cardiomyopathy, both of which are accompanied by absolute or relative decrease of oxygen supply to the heart muscle (Kokkonen et al 1999).

Recently, we have demonstrated that a non-specific inhibitor of NEP-enzyme, phosphoramidon, potentiated the endothelium-dependent coronary relaxation induced by bradykinin in a non-hypoxic model (Krassói et al 2000). Thiorphan ((DL-3-mercapto-2-benzylpropanoyl)-glycine) is a more specific inhibitor of NEP and decreased NEP activity in concentrations (10 μM or lower) shown to have no effect on other vasopeptidases such as endothelin-converting enzyme (ECE) and ACE (Roques et al 1980; Maguire et al 1997).

In this study, we performed experiments with thiorphan under normoxic and moderately hypoxic conditions in porcine isolated coronary artery.

Materials and Methods

Drugs

Drugs used and their sources were as follows: bradykinin-acetate, thiorphan and U46619 (9,11-dideoxy-11$_\alpha$,9$_\alpha$-epoxymethano-prostaglandin $F_{2\alpha}$) (Sigma, St Louis, MO). Bradykinin was dissolved in distilled water, thiorphan and U46619 in 96% ethanol. Until use, all the drugs were stored at 4 °C.

Organ preparation

Coronary arteries were obtained from porcine hearts that were harvested in a local abattoir. After removing the heart it was placed into ice-cold Krebs-Henseleit solution (composition in mM: NaCl 120; KCl 4.2; $CaCl_2$ 1.5; $NaHCO_3$ 20; $MgCl_2$ 1.2; KH_2PO_4 1.2; and glucose 11) and transported to the laboratory within 1 h. Coronary arteries of the circumflex branch were dissected free from the surrounding connective tissue and cut into rings, 5 mm long. Ring segments were mounted on a pair of stainless-steel hooks and placed into water-thermostated (at 37 °C) organ chambers containing 2 mL of Krebs-Henseleit solution. The solution was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 (pH 7.4). One of the hooks was anchored inside the organ chamber and the other was connected to a force-displacement transducer (Experimetria, Budapest, Hungary) to measure changes in isometric tension, as previously described (Krassói et al 2000). The rings were stretched up to 3 g, and equilibrated for 90 min. During this period, tension was continuously readjusted to the above value of stretch and the medium was changed in every 15 min. The partial pressure of oxygen (pO_2) was measured in the organ bath by a Clarck electrode (ISO-O_2, World Precision Instruments, Sarasota, Florida). Acute hypoxia was induced by changing 95% O_2 and 5% CO_2 bubbling to 95% N_2 and 5% CO_2 in 2 mL Krebs-Henseleit solution of the organ bath. All the experiments were performed using paired endothelium-intact vascular samples isolated from the same porcine heart. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals.

Effect of hypoxia on bradykinin-induced coronary relaxation

Endothelium-intact coronary artery rings, mounted parallel in separate organ chambers, were contracted with either 0.75 μM U46619 or 30 mM potassium chloride (KCl). When the contraction reached a stable plateau (usually after 10 min in both cases) one of the coronary rings was exposed to hypoxia, while the other one remained normoxic. Hypoxic change in isometric tension was stabilized 19.2 ± 2.0 min (in U46619 contracture) or 25.8 ± 1.5 min (in KCl contracture) after starting nitrogen bubbling and then bradykinin was administered cumulatively (1–1000 nM) into the organ baths. Hypoxia was then maintained for 31.2 ± 2.7 min (in U46619 contracture) or 41.1 ± 2.6 min (in KCl contracture), respectively.

Effect of the neutral endopeptidase inhibitor thiorphan on bradykinin-induced relaxation

In another series of experiments, the possible capacity of thiorphan to potentiate the bradykinin-induced relaxation was investigated under normoxic conditions. In each experiment, two parallel endothelium intact rings were mounted in separate organ baths. One of the coronary rings was pre-treated with thiorphan (10 μM), while the other one was pre-incubated with the corresponding volume of thiorphan solvent (20 μL of 96% ethanol). After 30 min, the rings ($n = 9$) were contracted with 0.75 μM U46619 and concentration–relaxation curves for bradykinin (1–1000 nM) were established. Coronary tissues derived from 9 other hearts were used for repeating the same protocol but the contractile agent was 30 mM KCl.

Effect of hypoxia on the relaxation induced by the combination of bradykinin and thiorphan

The following two series of experiments were devoted to study whether hypoxia was able to modify the endothelium-dependent vasoactivity of bradykinin in the presence of the NEP-inhibitor. In both groups of experiments, thiorphan was administered to one of the parallel coronary rings in a concentration of 10 μM, while the other one was pre-incubated with its solvent. Rings pre-contracted with either U46619 (first group) or KCl (second group) were made hypoxic as described before, except that each ring of the two parallel samples was exposed to nitrogen atmosphere. Thereafter, concentration–response curves were established by cumulatively added bradykinin. Acute hypoxia lasted for 27.3 ± 0.5 min (in U46619 contracture) or 40.8 ± 2.3 min (in KCl contracture).

Data analysis

Contractions induced by either 0.75 μM U46619 or 30 mM KCl were expressed in grams. Bradykinin-induced

decrease of coronary tone measured both at normoxic and hypoxic tensions was compared with the initial normoxic contraction amplitudes evoked by either U46619 or KCl. The effective concentration of bradykinin that caused 50% of maximal relaxation was defined as EC50. For the calculation of EC50 values, the $(a \times x)/(x + b)$ logistic equation was fitted to the individual dose–response values. From these fitted equations, the mean log EC50 value ± s.e.m. was calculated for each group.

Statistical analysis

All data are expressed as means ± s.e.m.. Two-way analysis of variance was used to assess the significance of differences between concentration–response curves. In case of significant effects, pairwise comparisons were corrected by Bonferroni-test. Student's two-tailed paired and unpaired *t*-tests were used to evaluate the effect of drug treatment on the EC50 values and on the changes in tone without bradykinin. *P* values less than 0.05 were considered statistically significant.

Results

Effect of acute hypoxia on the bradykinin-induced relaxation in porcine epicardial coronary artery

In Figure 1, the effect of acute hypoxia on bradykinin-induced relaxation in U46619 (A) and KCl (B) contractures is depicted. Hypoxia alone produced a transient increase followed by a sustained decrease both of U46619- and KCl-induced tension. The hypoxic tone became stable after 19.2 ± 2.0 min (U46619) and 25.8 ± 1.5 min (KCl) and was maintained until the end of the experiments (31.2 ± 2.7 min and 41.1 ± 2.6 min, respectively). At this time, hypoxia was detected as moderately severe ($pO_2 = 52.4 \pm 3.6$ mmHg (U46619) and 56.7 ± 4.1 mmHg (KCl)). Partial inhibition of bradykinin-induced vasorelaxation was achieved during hypoxia independently of the type of contractile agent and this inhibition proved to be significant over the entire concentration range of the endothelial stimulant (10–1000 nM bradykinin). The degree of the hypoxia-elicited diminution in bradykinin-induced relaxation was larger when the arteries were contracted with U46619. In the presence of U46619, hypoxic relaxation induced by bradykinin was not characterized by a significant change in the potency of bradykinin (normoxic EC50 = − 8.46 ± 0.25 log M vs hypoxic EC50 = − 8.07 ± 0.32 log M, n = 7). However, acute hypoxia significantly suppressed the maximum effect of bradykinin when KCl was used as contractile agent. This suggests a non-competitive interaction between hypoxia and the peptide.

Effect of thiorphan on bradykinin-induced relaxation in normoxic conditions

The effect of thiorphan was studied on bradykinin-induced coronary relaxation under normoxic conditions

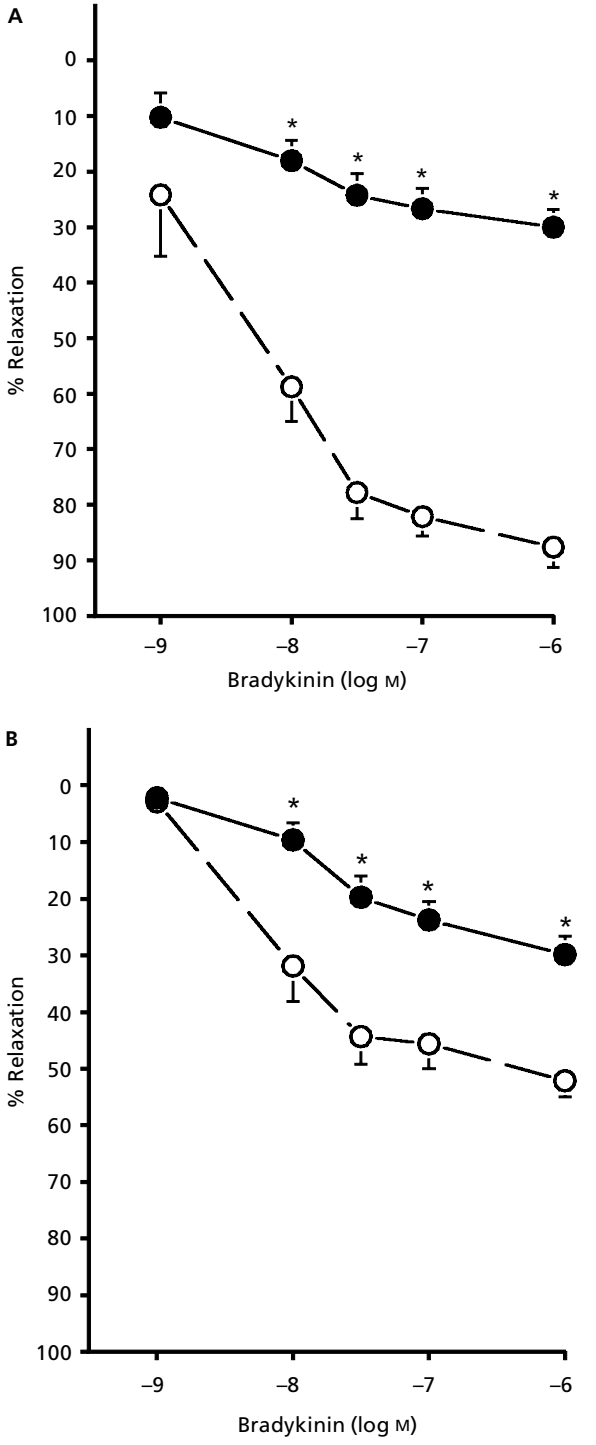

Figure 1 Effect of acute hypoxia on bradykinin-induced relaxation in porcine coronary artery rings. Ordinate indicates the relaxation of the vascular tissue expressed as % of the contraction induced by U46619 (0.75 μM) (A) or potassium chloride (30 mM) (B). Values are given as means ± s.e.m., ○ effect of bradykinin under normoxic conditions (A: $pO_2 = 98.8 \pm 7.2$ mmHg, n = 7; B: $pO_2 = 92.3 \pm 6.0$ mmHg, n = 7); ●, effect of bradykinin under moderately severe hypoxic conditions (A: $pO_2 = 52.4 \pm 3.6$ mmHg, n = 7; B: $pO_2 = 56.7 \pm 4.1$ mmHg, n = 7). *$P < 0.05$, normoxic vs hypoxic conditions.

in the presence of U46619 (Figure 2A) and KCl (Figure 2B) as contractile agents ($pO_2 = 101.5 \pm 8.0$ mmHg, U46619 and $pO_2 = 98.5 \pm 2.1$ mmHg, KCl). When either U46619 or KCl was applied, thiorphan tended to enhance the effect of bradykinin at low concentrations. The vasodilating effect of 1–10 nM bradykinin was significantly increased by the NEP-inhibitor following U46619 administration. However, these changes were not significant when KCl was used to depolarize the coronary artery smooth muscle. In the presence of U46619, the EC50 value of bradykinin in combination with thiorphan was significantly changed compared with bradykinin alone (bradykinin EC50 = -8.67 ± 0.20 log M, n = 9 vs bradykinin + thiorphan EC50 = -9.31 ± 0.19 log M, n = 9; $P < 0.01$).

Effect of thiorphan on bradykinin-induced relaxation under hypoxic conditions

Another set of experiments was devoted to explore whether thiorphan is able to modify the effect of bradykinin under moderately severe hypoxic conditions. The experimental protocol was entirely the same as described in the previous paragraph except that both coronary rings were exposed to nitrogen atmosphere (Figure 3). When U46619 was the contractile agent, acute hypoxia ($pO_2 = 48.6 \pm 5.7$ mmHg) was maintained for 27.3 ± 0.5 min (Figure 3A). Under these conditions thiorphan was unable to potentiate the bradykinin-induced relaxation and also did not significantly influence the EC50 value of the endothelial stimulant (bradykinin EC50 = -8.57 ± 0.32 log M vs bradykinin + thiorphan EC50 = -9.0 ± 0.18 log M, n = 8). Following contraction induced by KCl, hypoxia with a pO_2 value of 54.1 ± 2.4 mmHg lasted for 40.8 ± 2.3 min. In contrast to the results obtained with U46619, thiorphan clearly potentiated the relaxation induced by all concentrations of bradykinin applied. This enhancement of vasorelaxing effect was not followed by a significant shift in EC50 values (bradykinin EC50 = -8.15 ± 0.25 log M vs thiorphan EC50 = -8.58 ± 0.20 log M, n = 9).

Effect of thiorphan on the basal, U46619-elevated, KCl-elevated and hypoxic tones

We have evaluated the possible effect of thiorphan on the different levels of coronary tone calculated from the above three series of experiments. After 30 min pre-incubation of endothelium-intact coronary preparations with thiorphan, no marked differences in the in-vitro basal tone (3 g, see Materials and Methods) were observed compared with control either in U46619 (2.43 ± 0.23 g vs 2.60 ± 0.20 g, n = 8, respectively) or in KCl contracture (2.78 ± 0.05 g vs 3.03 ± 0.16 g, n = 9, respectively). Similarly, thiorphan also did not significantly affect the contraction when either U46619 (without thiorphan 8.78 ± 0.61 g vs thiorphan treatment 9.44 ± 3.84 g, n = 8) or KCl was applied as a contractile agent (without thiorphan 7.86 ± 0.73 g vs thiorphan treatment 6.80 ± 0.58 g, n = 9). Hypoxia first

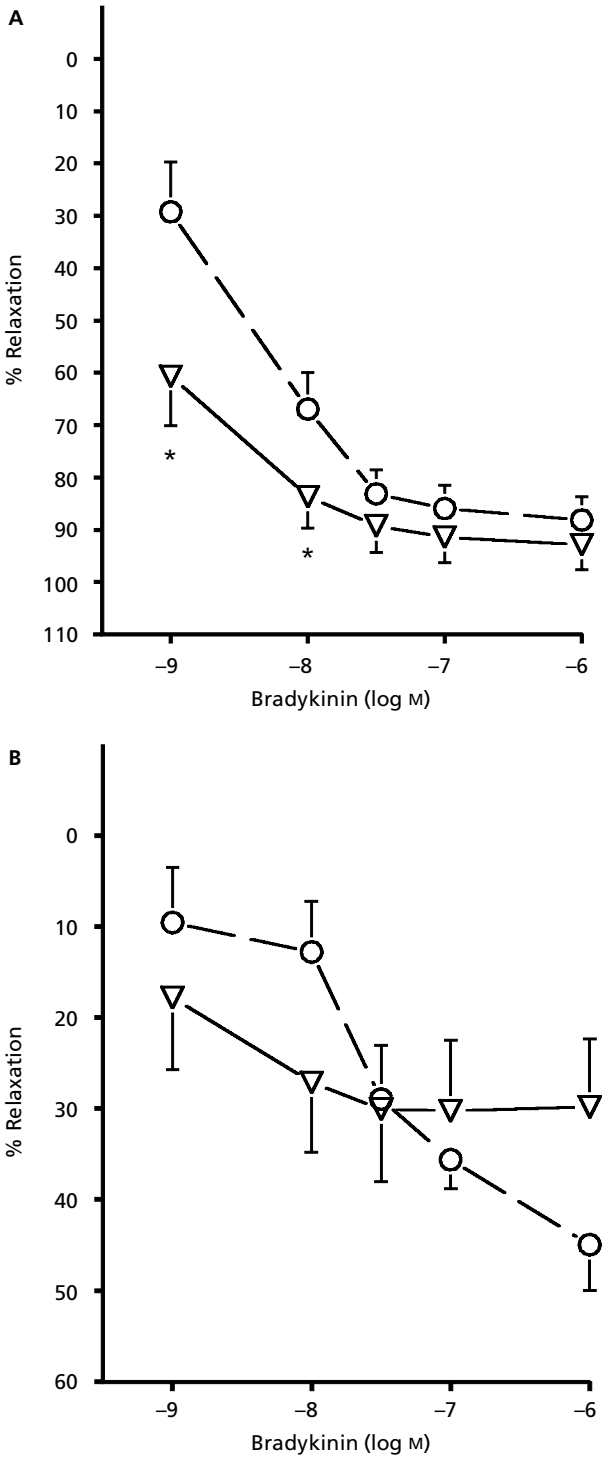

Figure 2 Effect of thiorphan (10 μM) on bradykinin-induced relaxation in porcine coronary artery rings under normoxic conditions. The vascular rings were contracted with 0.75 μM U46619 (A) or 30 mM potassium chloride (B). Ordinate depicts the magnitude of % relaxation expressed as in Figure 1. The symbols represent bradykinin-induced relaxation in the absence (○) or presence (▽) of thiorphan in normoxia (A: $pO_2 = 101.5 \pm 8.0$ mmHg, n = 9; B: $pO_2 = 98.5 \pm 2.1$ mmHg, n = 9). *$P < 0.05$, values obtained with vs without thiorphan.

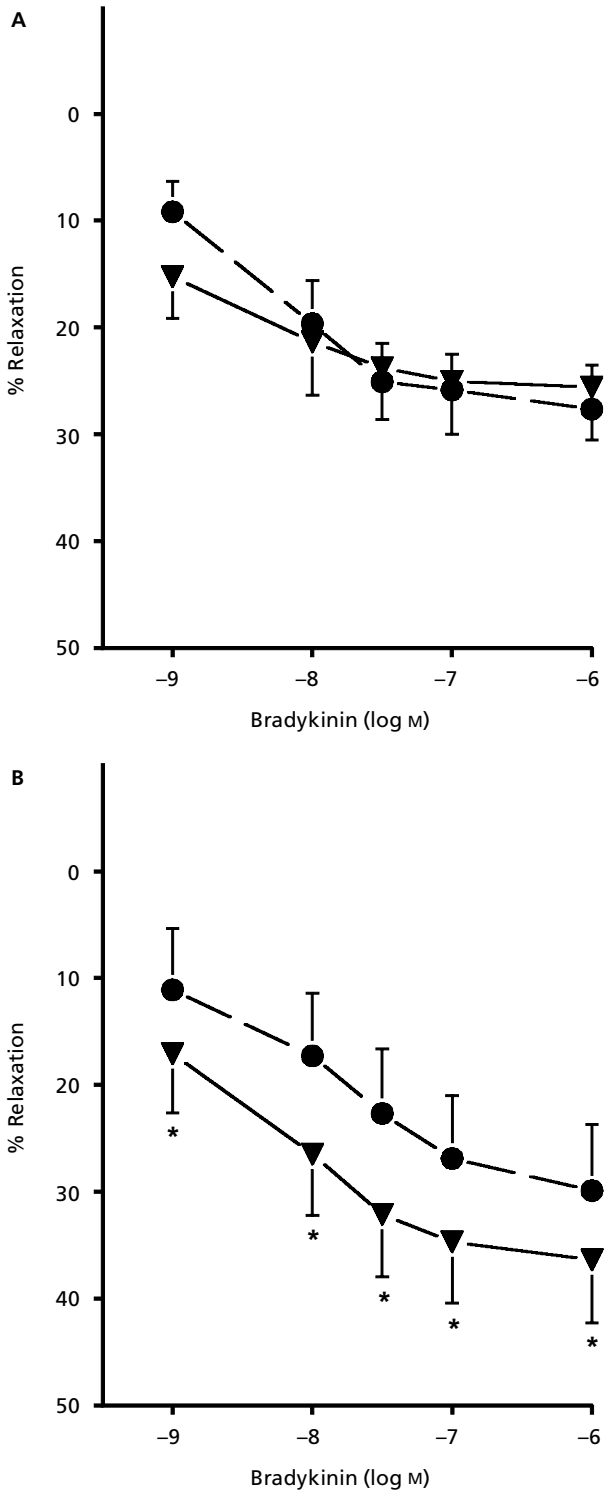

Figure 3 Effect of thiorphan (10 μM) on bradykinin-induced relaxation in porcine coronary artery rings under moderately severe hypoxic conditions. The vascular rings were contracted with 0.75 μM U46619 (A) or 30 mM potassium chloride (B). Ordinate depicts the magnitude of % relaxation expressed as in Figure 1. ●, effect of bradykinin alone; ▼, effect of bradykinin in combination with thiorphan in hypoxia (A: $pO_2 = 48.6 \pm 5.7$ mmHg, n = 8; B: $pO_2 = 54.1 \pm 2.4$ mmHg, n = 9). *$P < 0.05$, values obtained with vs without thiorphan.

produced a transient increase in both U46619- and KCl-stimulated tones. This transient increase in hypoxic tension was not considerably affected by thiorphan in either case (U46619 without thiorphan 0.32 ± 0.15 g vs with thiorphan 0.58 ± 0.32 g, n = 8; KCl without thiorphan 0.95 ± 0.20 g vs with thiorphan 0.94 ± 0.27 g, n = 9). The hypoxic contraction was followed by a sustained relaxation phase in the case of both contractile agents. The magnitude of this relaxation in U46619-contracted preparations was found to be 5.08 ± 0.9 g (n = 8), and in KCl contracture was 1.51 ± 0.34 g (n = 9), which again was not altered significantly by the NEP-inhibitor (5.47 ± 0.69, n = 8, and 1.97 ± 0.30 g, n = 9, respectively).

Discussion

In this study, one of the most important findings is that moderate hypoxia depresses the function of the coronary endothelium and decreases the vasorelaxation evoked by bradykinin. Similar studies were performed earlier under severe hypoxic conditions ($pO_2 < 20$ mmHg) (Vedernikov et al 1991; Shimizu & Paul 1999). However, such an excessive hypoxia usually does not occur at 37 °C in clinical settings such as heart surgery. There are only a few observations made in experimental circumstances when hypoxia was moderately severe. Our current experimental arrangement serves as a model for an acute moderate hypoxic episode ($pO_2 \sim 50$–60 mmHg, duration $\sim$ 50 min), which occurs as the consequence of the transfusion of diluted blood, cardiac arrest or a long-lasting period of apnoea following general anaesthesia (Siggaard-Andersen et al 1995). Our study is the first observation about the decrease of the coronary vasorelaxing effect of the important endothelial relaxant bradykinin in a model of moderately severe hypoxia. The deterioration of endothelial function could be observed independently of the mechanism of coronary constriction induced by prostaglandin (U46619) or depolarization (KCl) and it may contribute to the known perioperative increase of the vasospastic tendency in conduit-type coronary arteries.

Thiorphan, a specific inhibitor of NEP-enzyme, was able to enhance the effect of bradykinin under normoxic conditions only in the presence of U46619. In the normoxic medium, potentiation by thiorphan was evident only at, and below, a bradykinin concentration of 10 nM. The lack of positive interaction between the two substances at high concentrations of bradykinin can be theoretically explained. One possible reason is that, in porcine coronary artery, bradykinin produces free radicals in the presence of oxygen, and it was suggested that the activity of NEP-enzyme was affected by the free radical, superoxide anion (Hernanz et al 1999; Huang et al 2000; Fleming et al 2001). The higher the pO_2 value, the larger the amount of oxygen-derived free radicals that are formed. This is especially valid for those experiments where KCl was the contractile agent. Under these conditions, no consequent biological effect of thiorphan was observed. Thus it appears logical that

NEP-enzyme activity will less likely be changed by the above mechanism at lower oxygen tension. Indeed, potentiation of coronary artery relaxation by thiorphan was evident in the whole concentration range of bradykinin in hypoxic + hyperkalaemic solution. The effect of thiorphan may have significance in-vivo. The concentration of bradykinin was found to be 4.2 nM in human plasma (Verma et al 1980), which represents the lowest part of the concentration–response curves (i.e. 1–10 nM bradykinin) in that thiorphan–bradykinin interactions are evident in hypoxic/hyperkalaemic solution. This condition would represent the circumstances used, for example, in cardiac surgery.

In hypoxia, thiorphan potentiated the effect of larger concentrations of bradykinin only when the smooth muscle of the coronary arteries was depolarized (i.e. in the presence of KCl). This type of interaction could not be observed following U46619-induced contracture. We have two explanations for this. One is that the lack of potentiation by thiorphan may derive from the considerably lower hypoxic tone in the presence of U46619 compared with KCl. When U46619 contraction was followed by hypoxia, the coronary artery was relaxed by 58% and in the presence of KCl the tone was lowered by 19%. This difference could not be due to hyperkalaemia-induced decrease of the endothelial function (He & Yang 1996), but rather to an endothelium-independent and presently unknown mechanism observed recently by others (Shimizu et al 2000). The other reason for the lack of thiorphan-induced potentiation of bradykinin-induced relaxation may be due to decreased nitric oxide production in hypoxia (Shimizu & Paul 1999).

The available information on the exact role of NEP-enzyme, EC.3.4.24.11, in the physiological and pathological regulation of coronary circulation is scanty and further investigation is required in this area. The contribution of this enzyme to the degradation of bradykinin is established in kidney epithelial cells and in the interstitium of the human heart (Blais et al 2000). The only evidence for the role of NEP-enzyme in modulating the coronary vasomotor tone has been presented in the rat heart (Dumoulin et al 1998). Therefore our study provides the first functional evidence for the effect of thiorphan on coronary artery in a larger mammalian species.

The metabolism of bradykinin involves several different vasopeptidases, including aminopeptidases, carboxypeptidases and endopeptidases (Blais et al 2000). Among these enzymes, ACE and ECE are not influenced by thiorphan; it exhibits pharmacological characteristics similar to those of the recently discovered candoxatril, another NEP-inhibitor (Maguire et al 1997; Kentsch & Otter 1999; Blais et al 2000). The efficiency of thiorphan indirectly proves the significant role of a specific NEP-enzyme, EC.3.4.24.11, in the degradation of bradykinin in porcine coronary artery. In our experiments, thiorphan did not influence the tone of the coronary samples in the absence of exogenously added bradykinin, including the basal, KCl-elevated and hypoxia-induced tones. The latter findings also exclude a non-specific vascular effect of the NEP-inhibition by thiorphan.

Conclusion

Vasopeptidase inhibitors, including those that decrease the activity of the neutral endopeptidase enzyme (EC.3.4.24.11), are potential vasodilator drugs. Heart transplants are stored in high-potassium medium, and hypoxia is an important consequence of low cardiac output which frequently occurs in certain clinical settings, such as cardiac surgery. The inhibition of NEP-enzyme appears to improve the known deleterious effects of hyperkalaemia and hypoxia on the endothelial function. In hypoxia the inhibition of NEP-enzyme activity appears to have no effect when the potassium concentration is physiological. Therefore, the use of a NEP-inhibitor, such as thiorphan, would increase the relaxing capacity of depolarized coronary arteries, for example, in heart transplant surgery.

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