—Regular Article—

Role of the Nitric Oxide on Relaxation of the Human Umbilical Artery during Cooling

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In the present study, the effects of cooling (to 28℃) on the vasodilatation induced by diazoxide ($10^{-9}-3\times10^{-4}$ M), isoproterenol ($10^{-9}-3\times10^{-4}$ M) and magnesium sulphate (0.1–30 mM) on serotonin-pre-contracted human umbilical artery and the role of nitric oxide in these effects were analyzed. Diazoxide, isoproterenol and magnesium produced concentration-dependent relaxation of human umbilical artery precontracted with serotonin (10^{-6} M). During cooling, the pIC_{50} values and maximal responses to these agents were significantly lower than at 37℃. Cooling to 28 ℃ in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) did not modify the effects of temperature on diazoxide, isoproterenol and magnesium-induced relaxations. These results suggest that cooling-induced changes of diazoxide, isoproterenol, and magnesium sulphate in human umbilical artery are independent of nitric oxide.

Key words——cooling; diazoxide; isoproterenol; magnesium; nitric oxide; umbilical artery

INTRODUCTION

Responses of vascular smooth muscles caused by agonist and antagonists can be influenced by some physical factors such as temperature.[1] Lowering the temperature (cooling) has been shown to have a variable influence on vascular sensitivity to various drugs and endogenous substances in different parts of the vascular system of animals.[2,3] Most of the previous studies examining the effect of cooling on smooth muscle responses have focused on the effects of contractile agents and information about vasodilators is rather limited. Saito *et al.*[4] reported that in guinea-pig myocardium and aorta the vasorelaxant effects of K^+ channel openers, NIP-121, cromakalim, and pinacidil, were greatly reduced during cooling. Thus the investigators demonstrated that the effects of K^+ channel openers on the myocardium and vascular smooth muscle are temperature sensitive.

Endothelium serves as an important modulator of vascular smooth muscle tone, through the formation of several vasoactive species.[5] It has been proposed that in states of health, endothelium-derived substances that exhibit smooth muscle relaxant properties predominate.[6] Nitric oxide is a relaxant factor that produces a basal vasodilator tone and is involved in the response of different vascular beds to vasoactive stimuli. Nitric oxide is synthesized in the endothelium from L-arginine and this synthesis can be stimulated by several types of stimuli, and is inhibited by L-arginine analogues.[7] Limited evidence suggests that vascular smooth muscle responsiveness to nitric oxide and related factors can be influenced by temperature.[8–10]

Thus, despite current research to determine the effects of temperature on vascular reactivity of different animals species, studies with human tissues remain incomplete and very few have examined the effect of cooling on the smooth muscle dilatation of the human umbilical artery. The human umbilical artery is an unique mammalian artery that lacks autonomic innervation.[11] Thus, the vascular tone of human umbilical artery that modulates fetoplacental circulation, is regulated by local mediators such as prostaglandins and 5-HT or some ions such as potassium and calcium.[12,13]

The purpose of this study was to determine the effects of cooling on the vasodilatory effects of diazoxide, isoproterenol and magnesium on human umbilical artery *in vitro*. The umbilical artery was selected because it is easily accessible human vessel and is appropriate for studying the effects of these drugs.

METHODS

Human umbilical cords obtained from normal, spontaneous and full-term transvaginal deliveries

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were used in this study. Cords obtained from mothers with eclampsia, hypertension, diabetes or other overt diseases were not used for this study. Cords obtained from mothers on medication, such as uterotonic agents during labour, were also excluded and no analgesics were prescribed during labour. Maternal age ranged between 25 and 32 years.

Immediately after delivery the umbilical cords were placed in cold Krebs-Henseleit solution (KHS, mM: NaCl 119, KCl 4.70, $MgSO_4$ 1.50, KH_2PO_4 1.20, $CaCl_2$ 2.50, $NaHCO_3$ 25, Glucose 11). Umbilical arteries were carefully isolated from connective tissue and cut into 2×15 mm helical strips within 12 h of delivery. One artery was dissected from each cord. Care was taken not to injure the endothelium during the preparation. Strips were mounted in 25 ml organ baths containing KHS at 37℃ continuously gassed with 95% O_2 and 5% CO_2. At the beginning of the experiment, the strips were stretched to an initial tension of 1 g and allowed to equilibrate for 60 min in the KHS, which was changed every 15 min. Isometric contractions were recorded with an oscillograph (Harvard).

Before each experiment, to check the endothelial cell integrity, the preparations were contracted with 10^{-6} M 5-HT and then acetycholine (10^{-6} M) was added. Preparations which relaxed by more than 70% of the 5-HT-induced tone after addition of acetycholine were considered to have undamaged endothelium. After endothelium integrity tests, the preparations were washed and allowed to return the basal tension.

First, the preparations were contracted with 10^{-6} M 5-HT. After the contraction had reached steady state, diazoxide was added to the organ bath cumulatively ($10^{-9}-3\times10^{-4}$ M) at 37℃. After the first concentration-response curve was completed, preparations were washed and allowed to reestablish resting tension. After the contractile responses to 5-HT, the temperature was changed from 37 to 28℃ (cooling). Cooling was rapidly achieved and preparations were allowed to equilibrate at this temperature for 20 min before a second cumulative concentration-response curve was determined for diazoxide.

The influence of nitric oxide on relaxations to diazoxide was specifically addressed by pre-treating the strips with the nitric oxide synthase inhibitor N^G nitro-L-arginine methlyl ester (L-NAME, 10^{-4} M).[8] Again, after the contractile responses to 5-HT, the temperature was changed from 37 to 28℃. The tissues were allowed to equilibrate at 28℃ for 20 min. L-NAME was added to the organ bath 20 min before concentration-response curves were obtained. Endothelium was not denuded because only the role of endothelial nitric oxide was examined in this study.

The same procedures were repeated with isoproterenol ($10^{-9}-3\times10^{-4}$ M) and magnesium sulphate (0.1–30 mM) on 5-HT-pre-contracted preparations. Only one agent was tested in each preparation.

In another series of the experiments, the relaxant effect of sodium nitroprusside ($10^{-9}-3\times10^{-4}$ M) was investigated in preparations pre-contracted with 5-HT (10^{-6} M) at 37 and 28℃.

Statistical Analysis The maximal 5-HT contraction was used as a standard by which subsequent responses of the tissue could be expressed (as a percentage of this contraction). Relaxation responses to diazoxide, isoproterenol and magnesium are expressed as percentages of the 5-HT (10^{-6} M) induced contraction. Concentrations of diazoxide, isoproterenol and magnesium causing 50% of the maximal response (IC_{50}) were calculated from each individual concentration-response curve and its 95% confidence interval were obtained for each group of experiments. Maximal responses (E_{max}) and pIC_{50} ($-\log IC_{50}$) values for curves obtained before and during cooling were compared by using Student's t test. Statistical significance was set at $p<0.05$.

Drugs 5-Hydroxytryptamine creatinine sulphate (5-HT), NG nitro L-arginine methyl ester, sodium nitroprusside, histamine, isoproterenol (all dissolved in distilled water), diazoxide (dissolved in dimethyl sulphoxide; DMSO. The concentration of DMSO in the tissue bath was always kept below 0.4 %) were used. All drugs were obtained from Sigma (St. Louis, MO, USA).

RESULTS

Response to Diazoxide In human umbilical artery, 5-HT (10^{-6} M) produced reproducible contractions. After the maximal contractile response to 5-HT, cooling to 28℃ did not change this contraction. Maximal response (g) to 5-HT was found 1.3 ± 0.2 and 1.4 ± 0.1, at 37 and 28℃, respectively. Diazoxide ($10^{-9}-3\times10^{-4}$ M) produced concentration-dependent relaxation of human umbilical artery preparations pre-contracted with 5-HT at both 37 and 28℃ (cooling). Figure 1 shows the results with di-

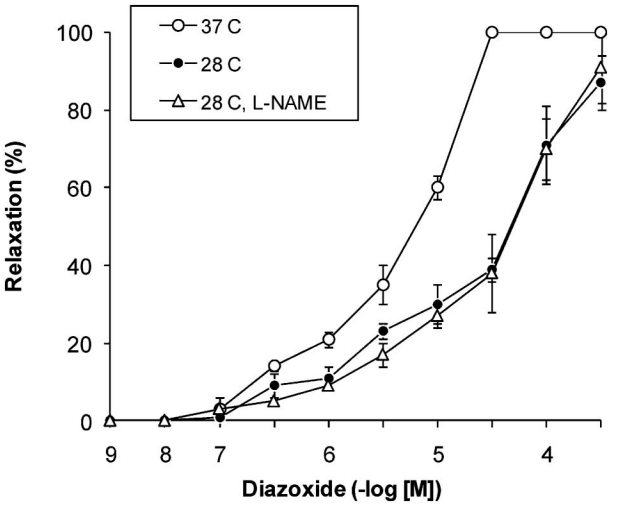

Fig. 1. Concentration-response Curves for Diazoxide at 37, 28℃ and in the Presence of L-NAME at 28℃, in Human Umbilical Artery

The preparations were pre-constricted with 5-HT (10^{-6} M). Each point is the mean±S.E.M. of six experiments.

azoxide in human umbilical artery at 37, 28 and also at 28℃ in the presence of L-NAME. Compared with 37℃, the pIC_{50} and E_{max} values of diazoxide were significantly lower ($p<0.05$) at 28℃ (Tables 1 and 2).

Pre-incubation with L-NAME did not significantly affect the effects of cooling ($p>0.05$; Table 1). L-NAME also did not affect the basal tone and the amplitude of the contraction induced by 5-HT in human umbilical artery.

Response to Isoproterenol Figure 2 shows the effects of isoproterenol ($10^{-9}-3\times10^{-4}$ M) at 37, 28 and also at 28℃ in the presence of L-NAME on human umbilical artery contracted with 5-HT (10^{-6} M). Cumulative addition of isoproterenol ($10^{-9}-3\times10^{-4}$ M) produced concentration-dependent relaxation of human umbilical arteries pre-contracted with 5-HT at both 37 and 28℃ (cooling). After the maximal contractile response to 5-HT, cooling to 28℃ did not change this contraction. During cooling the pIC_{50} and E_{max} values (Tables 1 and 2) of the human umbilical artery were significantly lower ($p<0.05$) than at 37℃.

Pre-treatment with L-NAME (10^{-4} M) did not alter the effects of temperature on isoproterenol-induced relaxation significantly ($p>0.05$; Table 1).

Response to Magnesium Sulphate Figure 3 shows the effects of magnesium sulphate (0.1–30 mM) at 37, 28 and also at 28℃ in the presence of L-NAME on human umbilical artery contracted with 5-HT (10^{-6} M). Cumulative addition of magnesium sulphate (0.1–30 mM) produced concentration-dependent relaxation of human umbilical arteries pre-contracted with 5-HT at both 37 and 28℃ (cooling). After the maximal contractile response to 5-HT, cooling to 28℃ did not change this contraction. During cooling the pIC_{50} and E_{max} values (Tables 1 and 2) of the human umbilical artery were significantly lower ($p<0.05$) than at 37℃.

Pre-treatment with L-NAME (10^{-4} M) did not alter the effect of temperature on magnesium sulphate-induced relaxation significantly ($p>0.05$; Table 1).

Effect of Sodium Nitroprusside Sodium nitroprusside-induced concentration dependent relaxation in human umbilical artery and the relaxation to this agent was not influenced by cooling (Fig. 4).

Table 1. pIC_{50} Values for Diazoxide, Isoproterenol and Magnesium Sulphate in Human Umbilical Artery at 37, 28℃ and 28℃ in the Presence of L-NAME

	pIC_{50}		
	Diazoxide	Isoproterenol	Magnesium sulphate
37℃ ($n=6$)	5.3±0.6	5.0±0.2	2.9±0.1
28℃ ($n=6$)	4.2±0.8*	4.0±0.8*	2.2±0.1*
28℃ L-NAME ($n=6$)	4.3±0.8	4.1±0.7	2.2±0.7

Each value is derived from six experiments. Data are means±S.E.M.
* $p<0.05$ compared to pIC_{50} values obtained at 37℃.

Table 2. Maximum Responses (E_{max}) for Diazoxide, Isoproterenol and Magnesium Sulphate in Human Umbilical Artery at 37, 28℃ and at 28℃ in the presence of L-NAME

	E_{max} (%)		
	Diazoxide	Isoproterenol	Magnesium sulphate
37℃ ($n=6$)	100±0.0	69.0±2.0	100±0.0
28℃ ($n=6$)	86.6±4.0*	57.0±4.0*	65.0±4.1*
28℃ L-NAME ($n=6$)	90.7±7.0	56.7±4.4	69.0±0.5

Each value is derived from six experiments. Data are means±S.E.M.
* $p<0.05$ compared to E_{max} values obtained at 37℃.

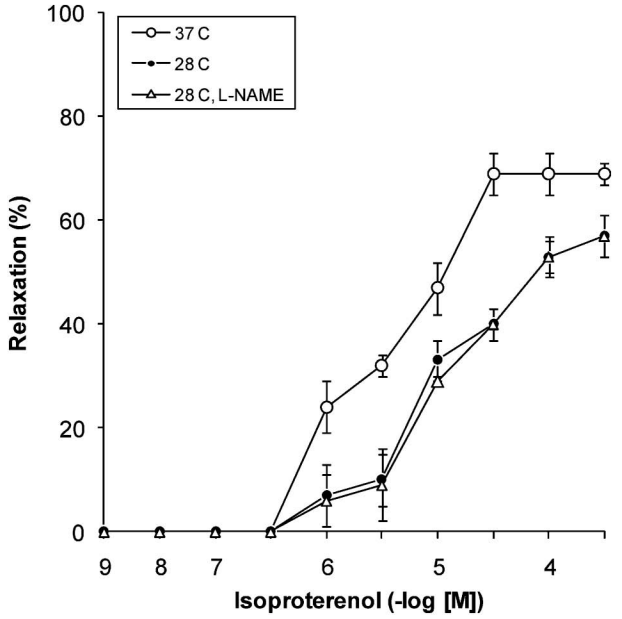

Fig. 2. Concentration-response Curves for Isoproterenol at 37 and 28°C and in the Presence of L-NAME at 28°C, in Human Umbilical Artery

The preparations were pre-constricted with 5-HT (10^{-6} M). Each point is the mean±S.E.M. of six experiments.

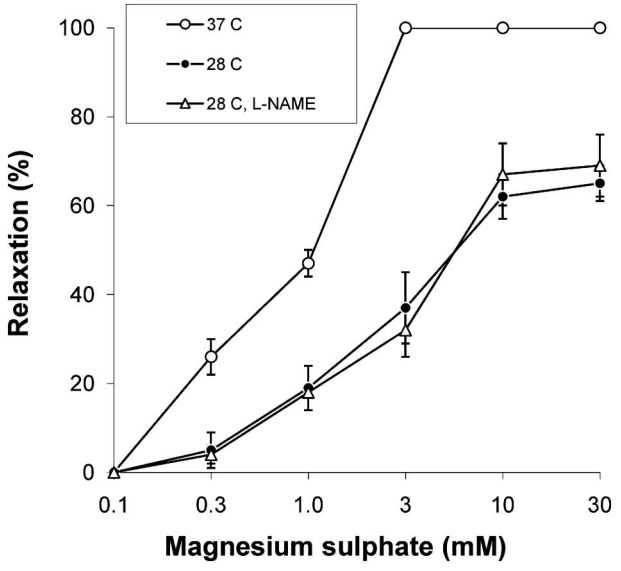

Fig. 3. Concentration-response Curves for Magnesium Sulphate at 37 and 28°C and in the Presence of L-NAME at 28°C, in Human Umbilical Artery

The preparations were pre-constricted with 5-HT (10^{-6} M). Each point is the mean±S.E.M. of six experiments.

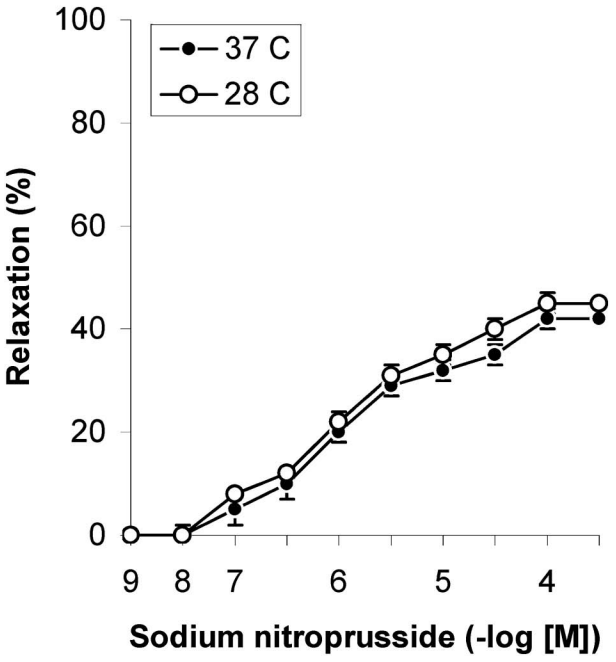

Fig. 4. Responses of Human Umbilical Artery to Sodium Nitroprusside at 37 and 28°C

Each point is the mean±S.E.M. of six experiments.

DISCUSSION

In the present work, we studied the effects of cooling on diazoxide, isoproterenol and magnesium sulphate-induced vasodilatation of human umbilical artery, paying special attention to the role of nitric oxide in these responses. The human umbilical artery is easily accesible smooth muscle preparations used frequently for functional studies but there is limited information[8,14,15] about the effect of temperature in this vessel and the effects of cooling were generally studied with contractile agents. Furthermore, to our

knowledge, there are no studies that analyze the effects of cooling on the diazoxide-, isoproterenol- and magnesium sulphate-induced relaxations.

The temperature utilized in this study; 28℃, for cooling was considered to be 'moderate cooling' temperature accordingly to our previous studies.[15–18]

Our results indicate that at 37℃, 5-HT-induced reproducible contractions in human umbilical artery. It is reported that in human umbilical vessels, 5-HT is the most potent agonist.[19] At 37℃, diazoxide induced concentration-dependent vasodilatation in preparations pre-contracted with 5-HT. Diazoxide directly dilates the vascular smooth muscle, by activation of ATP-sensitive K^+ channels.[20] K_{ATP} opening drugs are potent in reducing arterial pressure, and have been shown experimentally to relax blood vessels in the systemic, coronary and pulmonary circulations, indicating the presence of these channels.[21] Newgreen *et al.*[22] reported that diazoxide hyperpolarized the rat portal vein and relaxed 80 mM KCl induced contraction of rat aorta by opening of ATP-sensitive K^+ channels. In our study, compared with the control responses at 37℃, cooling decreased the relaxation to diazoxide. Beta-adrenoceptor agonists such as isoprenaline are known to produce vasorelaxation through activation of adenylyl-cyclase largely in an endothelium-independent manner and a subsequent increase in smooth-muscle cAMP levels.[23] In our study, isoproterenol induced concentration-dependent vasodilatation in preparations precontracted with 5-HT and compared with the control responses at 37℃, cooling decreased the relaxation to isoproterenol.

Studies on isolated human umbilical arteries and veins have shown inhibitory effects of magnesium, possibly based on inhibition of transmembrane calcium influx, and vasoconstriction secondary to deficiency of the ion has been proposed as an important factor in the increased placental vascular resistance seen in pre-eclampsia.[24–26] In our study, magnesium sulphate induced concentration-dependent vasodilatation in preparations precontracted with 5-HT and compared with the control responses at 37℃, cooling decreased the sensitivity, but not maximal relaxation to magnesium sulphate in this vessel. With our data we cannot suggest which mechanisms underlie the decreased sensitivity to diazoxide, isoproterenol and magnesium sulphate during cooling. Our results are in agreement with those reported by Tiritilli[3] who has reported that cooling decreased nicorandil, a ATP-sensitive K^+ channel-opener, induced relaxation in umbilical arteries. No previous data on the effects of diazoxide, isoproterenol and magnesium sulphate of human umbilical artery during cooling have been published. In our previous study, cooling increased the sensitivity to both 5-HT and ACh and the contractile responses of the umbilical artery and vein to either cooling or warming were in agreement with that of the cutaneous vessels of different species.[15] On the other hand, there are limited and conflicting reports about the effects of cooling due to using vasodilator agents and tissues. For example, Fernandez *et al.*[27] reported that cooling increased the sensitivity of the relaxation of the central ear artery, a cutaneous vessel, but it did not affect the response of the femoral artery, a noncutaneous vessel, to histamine. It has also been shown that the relaxation to cholinergic stimulation of ear artery, but not of femoral artery, from rabbit was increased during cooling.[28] The findings in guinea-pig myocardium and aorta support our results; Saito *et al.*[4] reported that the vasorelaxant effects of K^+ channel openers, NIP-121, cromakalim, and pinacidil, were greatly reduced during cooling. However, the mechanism underlying the effect of cooling to different agents is not clear.

In the vasculature, nitric oxide is physiologically important for maintaining vascular homeostasis, it keeps the vessels dilated, protects the intima from platelet aggregates and leukocyte adhesion, and prevents proliferation and migration of smooth muscle cells.[29] Although efforts are currently being made to understand the regulation, production and function of endothelial nitric oxide, its role in the effects of cooling on vascular reactivity has been little studied.[30,31] Limited data suggest that changing temperature may also alter the ability of the endothelium to generate or release nitric oxide.[30,31] In this study, we also investigated the role of nitric oxide in cooling induced responses to diazoxide, isoproterenol and magnesium sulphate. At 28℃, inhibition of nitric oxide synthesis did not modify the decreased relaxation to diazoxide, isoproterenol and magnesium sulphate, thus suggested that during cooling the responses to these agents are not modulated by nitric oxide. With our data we cannot suggest which mechanisms underlie this decreased sensitivity during cooling. Our results are in agreement with those reported by Tritilli,[3] who has shown that cooling

decreases nicorandil relaxation in umbilical arteries and nitric oxide pathway appears relatively unimportant, as pretreatment with L-arginine or the inhibition of nitric oxide-synthase by L-NNA did not modify the relaxation response to nicorandil. Furthermore, Bodellson *et al.*[31] reported that cooling specifically alters the sensitivity to various drugs in different parts of the vascular systems; in the rat jugular vein, cooling markedly augments the contraction to 5-HT and it has been found that this augmentation is dependent of an intact endothelium. Monge *et al.*[28] reported that the relaxations of ear arteries, but not of femoral arteries, from rabbits to cholinergic stimulation is increased during cooling, probably by an increased production of nitric oxide in the activated endothelium at low temperature. It is also reported that the increased sensitivity of the relaxation in ear arteries, but not in femoral arteries, to histamine found during cooling seems to be independent of the release of nitric oxide.[27] Recent studies in humans have shown that the sustained vasoconstrictor responses during local skin cooling is reduced by an nitric oxide synthase inhibitor, suggesting the involvement of nitric oxide synthase inhibition by cooling in the response.[32,33] However, no studies have analyzed the role of nitric oxide on diazoxide, isoproterenol and magnesium sulphate induced relaxation during cooling of human umbilical artery.

In our study, the effects of cooling to sodium nitroprusside, which mediates its vasorelaxant effects in contractile cells is *via* nitric oxide released in the vascular smooth cells[34] were also studied, and it was found that the relaxations to this substance were not influenced by cooling and it can therefore be assumed that the ability of the smooth muscle cells to relax to nitrous compounds remains essentially unaltered at lowered temperatures in human umbilical artery. In a previous study, we found the similar result with sodium nitroprusside in calf coronary artery and cardiac vein.[18]

In conclusion, the present results suggest that in human umbilical artery, during cooling nitric oxide has no role on the decreased sensitivity to diazoxide, isoproterenol and magnesium sulphate because endothelial nitric oxide did not affect the action of cooling. Further studies must be performed to clarify the mechanism of cooling-induced decrease in the response of these agents.

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