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Original article

# Normothermic and hypothermic models for studying the deleterious effects of hypoxia-reoxygenation on EDHF-mediated relaxation in isolated porcine coronary arteries

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ABSTRACT

**Introduction:** The vasomotor response of the coronary artery is altered by hypoxia–reoxygenation (H–R) induced damage. The aim of our study was to compare and evaluate normothermic and hypothermic models which are suitable for future drug studies of vasoprotective action against H–R injury. **Methods:** Porcine coronary arterial rings were isolated and placed in Krebs–Henseleit (K–H) solution. Rings were exposed to normoxic conditions (control group) and two different H–R conditions: the first induced by a 95% $N_2$–5% $CO_2$ gas mixture (40- and 60-min hypoxia) in a normothermic protocol, and the second induced by hypothermic (4 °C) hypoxia–reoxygenation in an air-tight beaker filled with K–H solution (24- and 48-hours hypoxia). Reoxygenation was applied by introducing K–H solution aerated with a 95% $O_2$–5% $CO_2$ mixture under normothermic (37 °C) conditions. To test the EDHF-mediated relaxation by substance P, rings were first incubated in L-NNA, nitric oxide synthase inhibitor, and indomethacin, cyclooxygenase inhibitor, and then pre-contracted with thromboxane analogue U-46619. Analysis of the maximum relaxation of the arterial rings was performed by one-way ANOVA, followed by Bonferroni's post-test. **Results:** Distal segments of the coronary artery responded faster to contraction induced by U-46619 and were relaxed by substance P to a greater extent than proximal segments. Maximal relaxations of arterial rings induced by a 10 nM solution of substance P were significantly reduced ($p<0.001$) from the values for normoxic rings (81.0±1.0%, $n=30$) after 40-min H–R (50.5±5.3%, $n=30$), 60-min H–R (32.1±3.5%, $n=30$), 24-hours hypothermic H–R (56.0±2.3%, $n=30$) and after 48-hours hypothermic H–R (38.5±5.1%, $n=30$). **Conclusions:** The model employing 40-min normothermic H–R is as effective as 24-hours hypothermic H–R, and 60-min normothermic H–R as 48-hours hypothermic H–R for studying the deleterious effects of H–R on EDHF-mediated relaxation.



## 1. Introduction

The performance of blood vessels during and/or after hypoxia–reoxygenation (H–R) injury is of great importance in various clinical conditions, such as myocardial infarction, atherosclerosis, lower oxygen partial pressure, peripheral artery disease, etc. Arteries deliver nutrition to cells and veins remove their metabolic products, thus maintaining an adequate microenvironment for functioning. Prolonged hypoxia leads to an impairment of vascular function (Laude, Thuillez, & Richard, 2001). The direct effects on cells are attributed to disablement of cellular ATP production, cell membrane depolarization and disruption of homeostatic processes regulating the intracellular calcium concentration (Saikumar, Dong, Weinberg, & Venkatachalam, 1998). Further injury occurs during reperfusion due to oxidative stress, which damages mitochondrial membranes and leads to cell death (Garg, Hofstra, Reutelingsperger, & Narula, 2003). In this process of hypoxia–reoxygenation (H–R) injury, both vascular endothelial and smooth muscle cells may be injured. Endothelial cells are affected first, which can be characterized by the loss of endothelium-dependent vasorelaxation (Kaeffer et al., 1996; Laude et al., 2001). However, the response to endothelium-independent vasodilators such as NO donors SIN-1 or nitro-prusside is intact after H–R injury (Laude, Beauchamp, Thuillez, & Richard, 2002; Richard, Kaeffer, Tron, & Thuillez, 1994; VanBenthuysen, McMurtry, & Horwitz, 1987). Moreover, the vasoconstriction appears to be resistant to hypoxia. This was confirmed in a study where contraction of porcine coronary artery rings induced by KCl or U-46619 was not significantly altered by approximately 20 min of oxygen deprivation (Thorne, Shimizu, & Paul, 2001). These studies suggest that the deleterious effects of H–R injury are expressed mainly on the endothelial cells, while smooth muscle cells appear to be more resistant to hypoxia.

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Endothelial cells play an important role in controlling local vascular tone, with the release of nitric oxide (NO), prostacyclin ($PGI_2$) or the as yet unidentified endothelium-derived hyperpolarizing factor (EDHF). The relative contributions of NO, $PGI_2$, and EDHF in regulating vascular tone have not been fully elucidated. However, several in vitro studies indicate that the importance of EDHF for endothelium-dependent relaxation increases as vascular size decreases (Hill, Phillips, & Sandow, 2001; Shimokawa et al., 1996). Although the molecular identity of EDHF has not been determined, there is evidence that EDHF causes hyperpolarization of the underlying vascular smooth muscle cells by opening $Ca^{2+}$-sensitive $K^+$ channels ($K_{Ca}$). The resulting hyperpolarization inhibits voltage-dependent $Ca^{2+}$ channels and subsequently produces relaxation of blood vessels due to the reduction of intracellular free $Ca^{2+}$ (Cheung, Chen, MacKay, & Burnette, 1999; Cohen & Vanhoutte, 1995; Komori & Vanhoutte, 1990). Reduction or failure of EDHF may result in increased local vascular resistance and in endothelial dysfunction of the small vessels (Matsumoto, Miyamori, Kobayashi, & Kamata, 2006).

Various experimental models are used in functional studies of H–R injury to isolated arteries. Experiments *in vitro* are performed on either isolated blood vessels or endothelial cell and smooth muscle cell cultures. The different responses appear to depend not only on the vascular bed and the species (human, monkey, dog, rat, pig, sheep, guinea pig), but also on the degree of hypoxia (Herrera & Walker, 1998). Although it is not clear which animal model may be most similar to man, the use of large mammals, like pigs, may have some advantages due to their anatomical and physiological characteristics. Porcine coronary arteries are readily available from local abattoirs and pigs are bred under controlled conditions. Therefore porcine coronary arteries are often used in hypoxic studies *in vitro*. Apart from the model itself, the studies are performed in the absence/presence of different channel antagonists, enzyme inhibitors, and various vasoconstricting (e.g. KCl, U-46619, prostaglandin F2α) and vasorelaxing (e.g. bradykinin, adenosine, substance P) agents. Relaxation induced by substance P is mediated primarily via NO and EDHF, while $PGI_2$ plays only a minor role (Shimizu & Paul, 1999). Our previous studies (Kuzner, Drevensek, Gersak, & Budihna, 2004), together with some others (Ren et al., 2001), have shown that H–R injury reduces the non-NO- and non-$PGI_2$-mediated (EDHF-mediated) relaxation induced by substance P in the porcine coronary arteries.

The results of different studies show specific changes in the vasomotor activity after exposure to H–R. Nevertheless the extrapolation of such diverse studies is difficult and therefore it is necessary to establish a uniform model for H–R injury studies. In the present study, we have investigated normothermic and hypothermic methods of inducing H–R injury to isolated porcine coronary arteries and their consequent vasorelaxation responses. Due to the expected importance of EDHF for endothelium-dependent relaxation in smaller arteries, including coronary arteries, vasorelaxation by the EDHF pathway was used to assess H–R models.

## 2. Methods

All experiments were conducted in accordance with permission issued by the Veterinary Administration of the Republic of Slovenia (permit SI-No.323-02-234/2005/2 and 323-02-232/2005/2).

### 2.1. Chemicals

Substance P and N(ω)-nitro-L-arginine (L-NNA) (both from Sigma-Aldrich Chemie, Steinheim, Germany) were dissolved in distilled water. Indomethacin (Sigma-Aldrich Chemie, Steinheim, Germany) was dissolved in ethanol. Thromboxane analogue 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U-46619) (Alexis, Lausen, Switzerland) was dissolved in dimethylsulfoxide (DMSO). The final concentrations of ethanol and DMSO in the organ baths were 0.1% and 0.03%, respectively. Vehicle control studies indicated that these concentrations of ethanol and DMSO have no effect on arterial function.

### 2.2. Preparation of arterial rings

Fresh porcine hearts (300–400 g in weight) obtained from a local abattoir were rinsed of blood and immediately placed in a container filled with cold (4 °C) Krebs–Henseleit solution (K–H) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, and glucose 11 (all Merck, Darmstadt, Germany). The left anterior descending (LAD) coronary artery was dissected, cleaned of fat and connective tissue, and cut into cylindrical rings (3–4 mm in length). Coronary arterial rings were divided into two groups according to the position of dissection. Those dissected 5–15 mm from the origin of the LAD coronary artery were considered to be proximal arterial rings, while those dissected 15–35 mm from the origin were considered as distal arterial rings. The first 5 mm of the artery served as the grasping surface for the forceps in fixing the artery during dissection. The endothelium was preserved by cautious dissection of the rings. In endothelium-denuded rings, the endothelium was removed mechanically by a wooden stick (Uluoglu & Zengil, 2003; Vedernikov, Graser, & Li, 1990).

### 2.3. Study protocols

Aortic rings were divided into the control group (exposed to normoxic conditions) and two study groups exposed to different H–R conditions, the first induced by a 95% $N_2$–5% $CO_2$ gas mixture (40- and 60-min hypoxia) in a normothermic protocol, and the second induced by hypothermic (4 °C) hypoxia-reoxygenation in an air-tight beaker filled with K–H solution (24- and 48-hours hypoxia) (Fig. 1B and 1C). Two 50 μm stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer to measure tension in the vessel, while the other was fastened to the stainless steel support block.

Rings from the control group and those from the normothermic hypoxia group were mounted immediately after dissection in standard organ baths filled with K–H solution, maintained at 37 °C and continuously bubbled with a 95% $O_2$–5% $CO_2$ mixture (Messer, Ruše, Slovenia). After mounting, rings were allowed to equilibrate at 5.0 g (50 mN) resting tension for 60 min and then contracted three times with 60 mM KCl to achieve stable contractions. On the other hand, rings from the hypothermic hypoxia group were first exposed to hypoxic conditions and then mounted in standard organ baths under the same conditions. Vascular responses were processed and recorded on a Dewetron acquisition system (Dewe-book-8, Dewetron, Graz, Austria) after analogue-digital conversion (National Instruments, NI PCI-6013, Austin, USA) on the hard disk of a personal computer by DeweSoft 6.0 software (Dewetron, Trbovlje, Slovenia).

In the *control group (normoxic conditions),* proximal and distal arterial rings were aerated with a mixture of 95% $O_2$–5% $CO_2$ (oxygenated K–H) for 60 min (Fig. 1A). The bath solution was considered oxygenated when the concentration of dissolved oxygen in the solution was above 25 mg/L as measured by an oxymeter (Greisinger electronic GmbH, Regenstauf, Germany).

In the *normothermic hypoxia group*, distal arterial rings were incubated in K–H solution aerated with a 95% $N_2$–5% $CO_2$ mixture, which decreased the bath $Po_2$ to <1% (Hashimoto et al., 1993), for either 40 or 60 min. The bath solution was considered hypoxic when the concentration of dissolved oxygen in the solution was below 2 mg/L. Arterial rings were then rinsed and incubated in K–H solution aerated with a 95% $O_2$–5% $CO_2$ mixture for 60-minute reoxygenation (Fig. 1B).

In the *hypothermic hypoxia group*, distal arterial rings were incubated for 24- and 48-hours at 4 °C in a glass beaker filled to the top with non-aerated K–H solution and made airtight with Parafilm