Helium Induces Preconditioning in Human Endothelium *In Vivo*

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ABSTRACT

Aims: Helium protects myocardium by inducing preconditioning in animals. We investigated whether human endothelium is preconditioned by helium inhalation *in vivo*.

Methods and Results: Forearm ischemia–reperfusion (I/R) in healthy volunteers (each group n = 10) was performed by inflating a blood pressure cuff for 20 min. Endothelium-dependent and endothelium-independent responses were measured after cumulative dose–response infusion of acetylcholine and sodium nitroprusside, respectively, at baseline and after 15 min of reperfusion using strain-gauge, venous occlusion plethysmography. Helium preconditioning was applied by inhalation of helium (79% helium, 21% oxygen) either 15 min (helium early preconditioning [He-EPC]) or 24 h before I/R (helium late preconditioning). Additional measurements of He-EPC were done after blockade of endothelial nitric oxide synthase. Plasma levels of cytokines, adhesion molecules, and cell-derived microparticles were determined. Forearm I/R attenuated endothelium-dependent vasodilation (acetylcholine) with unaltered endothelium-independent response (sodium nitroprusside). Both He-EPC and helium late preconditioning attenuated I/R-induced endothelial dysfunction (max increase in forearm blood flow in response to acetylcholine after I/R was 180 ± 24% [mean ± SEM] without preconditioning, 573 ± 140% after He-EPC, and 290 ± 32% after helium late preconditioning). Protection of helium was comparable to ischemic preconditioning (max forearm blood flow 436 ± 38%) and was not abolished after endothelial nitric oxide synthase blockade. He-EPC did not affect plasma levels of cytokines, adhesion molecules, or microparticles.

Conclusion: Helium is a nonanesthetic, nontoxic gas without hemodynamic side effects, which induces early and late preconditioning of human endothelium *in vivo*. Further studies have to investigate whether helium may be an instrument to induce endothelial preconditioning in patients with cardiovascular risk factors.

What We Already Know about This Topic

- Endothelial dysfunction is an independent risk factor for cardiovascular events, and endothelium-dependent vasodilation assessed before and after a brief period of ischemia may be used to evaluate the potential efficacy of therapeutic interventions

What This Study Tells Us That Is New

- Preconditioning with the noble gas, helium, protected endothelium against injury (measured by the magnitude of vasodilation in response to acetylcholine before *vs.* after forearm ischemia and reperfusion)
- The results suggest that helium might be an effective means of protecting patients against ischemia and reperfusion injury

⊕ Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

ISCHEMIC preconditioning (IPC) results in protection of organs against ischemia–reperfusion (I/R) injury by short, nonlethal periods of ischemia.[1] Two phases are distinguished, an early phase of protection induced by a stimulus directly before I/R, known as classical or early preconditioning, and a second window of protection that arises 12–72 h after administration of the stimulus, which is known as late preconditioning. Next to ischemia, pharmacologic agents (*i.e.*, volatile anesthetics)[2] can also induce preconditioning. The endothelium circumvents all blood vessels and serves as a first-line

Table 1. Demographics and Baseline Data of Randomized Volunteers

	Controls	I/R	He-EPC	He-LPC	IPC
Male/Female	5/5	6/4	6/4	6/4	5/4
Age, yr	24 ± 6	22 ± 3	27 ± 8	23 ± 5	23 ± 5
BMI	23.7 ± 4.2	22.2 ± 2.6	22.5 ± 3.0	21.6 ± 1.3	23.8 ± 4.2
SBP, mm Hg	126 ± 14	124 ± 17	121 ± 9	118 ± 14	122 ± 7
DBP, mm Hg	75 ± 7	73 ± 8	73 ± 9	70 ± 6	72 ± 8
ESR, mm/hr	3.7 ± 2.2	4.3 ± 4.9	4.3 ± 5.0	3.8 ± 2.4	3.6 ± 1.5
Heart rate, beats/min	58 ± 8	60 ± 9	62 ± 8	63 ± 10	67 ± 12
Hemoglobin, mg/dl	13.4 ± 2.2	13.5 ± 1.3	13.7 ± 1.3	14.3 ± 1.1	13.8 ± 1.1
Thrombocytes (10^9/l)	228 ± 24	230 ± 48	234 ± 61	220 ± 31	261 ± 56
Leukocytes (10^9/l)	5.2 ± 0.8	6.4 ± 3.2	5.8 ± 1.5	5.7 ± 1.0	6.3 ± 1.9

All data are expressed as mean ± SD. No statistical differences were observed between groups.

BMI = body mass index; controls = control group without ischemia; DBP = diastolic blood pressure; ESR = erythrocyte sedimentation rate; He-EPC = helium early preconditioning group; He-LPC = helium late preconditioning group; IPC = Ischemic preconditioning group; I/R = ischemia–reperfusion group; SBP = systolic blood pressure.

defense mechanism against organ and tissue injury. The protective functions of the endothelium include anticoagulation, antiinflammation, prevention of platelet activation, regulation of permeability, and regulation of vascular tone. I/R elicits profound changes in the endothelial homeostasis, as attested by a significant suppression of endothelium-dependent vasodilation.[3] Endothelial dysfunction is regarded as an independent risk factor for cardiovascular events[4] and is a surrogate marker for monitoring the efficacy of therapeutic strategies.[5,6]

Postischemic endothelial dysfunction can be attenuated by IPC, as was shown in a human forearm model using venous occlusion plethysmography. In this study, 20 min of forearm I/R resulted in a blunted vasodilatory response to acetylcholine, which could be restored by IPC.[3]

Animal studies demonstrated that the noble gas helium induces early[7] and late[8] preconditioning of the heart. Because helium is readily available, easy to administer, and has no known side effects, it has the potential to become a perfect preconditioning agent.

Forearm I/R induces endothelial dysfunction by reducing vasodilation induced by increasing doses of aceylcholine.[3] Our primary hypothesis is that helium preserves postischemic endothelial function.

There are markers of endothelial function present in plasma. Activated endothelial cells release nitric oxide products, inflammatory cytokines, adhesion molecules, regulators of hemostasis, and microparticles.[9] Microparticles are vesicles circulating in plasma, which are derived from various cells in response to cell activation, injury, or apoptosis. Endothelial microparticles have been used as a clinical and quantitative marker of endothelial cell dysfunction,[10] and their presence is inversely associated with acetylcholine-induced vasodilation in coronary arteries.[11] Our secondary hypothesis is that the underlying mechanism of helium preconditioning might be related to endothelial nitric oxide synthase (eNOS)

production, circulating cytokines and adhesion molecules, or cell-derived microparticles released after I/R.

Materials and Methods

The Institutional Review Board of the Academic Medical Center, Amsterdam, The Netherlands, approved the trial,** which was conducted in accordance with the International Conference on Harmonization on Good Clinical Practice Guidelines and the Declaration of Helsinki. All subjects gave written informed consent.

Subjects

A total of 58 healthy volunteers were included (baseline characteristics and demographics are shown in table 1). Volunteers abstained from caffeine, alcohol, and smoking 12 h before onset of the experiment. All experiments were performed in a quiet, temperature-controlled (20–24°C) room. Fifty volunteers were randomized to one of five groups using randomization software (ALEA; Nederlandse Kanker Instituut, Amsterdam, The Netherlands) provided by Clinical Research Unit of the Academic Medical Centre. An additional group of eight volunteers received the eNOS blocker N^G-monomethyl-L-arginine (L-NMMA).

Study Design

The study protocol is outlined in figure 1. Forearm ischemia was induced by inflating a 12-cm-wide blood pressure cuff placed on the nondominant upper arm to a pressure of 200 mm Hg for 20 min. Helium preconditioning was induced by administration of a helium mixture (Heliox: Helium 79%, Oxygen 21%, BOC, Mordon, United Kingdom) using a noninvasive delivery system (Helontix Vent; Linde Therapeutics, Eindhoven, The Netherlands) *via* a normal facemask with pressure support of 3 cm H_2O. Volunteers were given three cycles of helium for 5 min, followed by 5 min of normal air breathing either directly (helium preconditioning [He-EPC]) or 24 h (helium late preconditioning) before I/R (see fig. 1).

**Registered at www.trialregister.nl with number NTR1124. Accessed March 16, 2012.
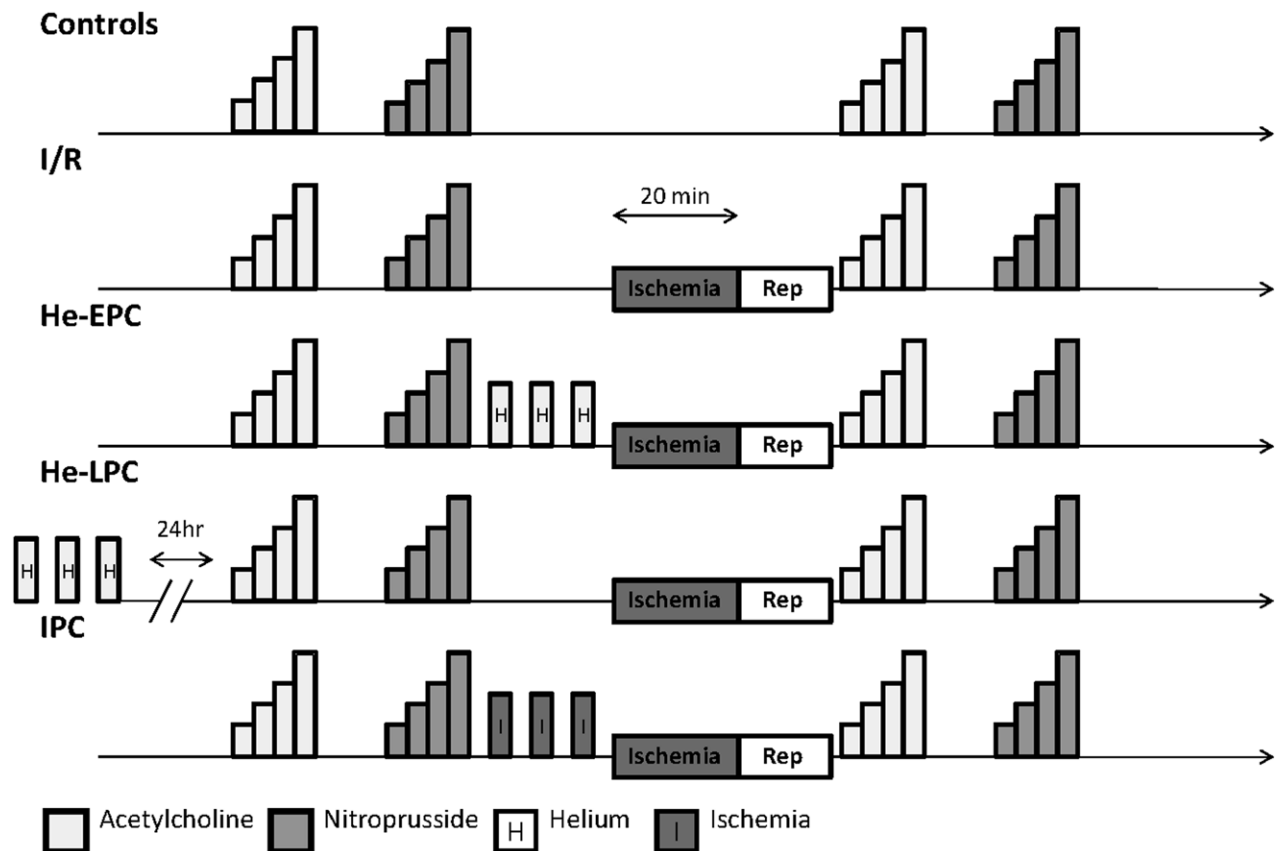
Fig. 1. Study protocol. Endothelial function was established at baseline and was repeated after 20 min of forearm I/R except for controls. Treatment with helium preconditioning was administered directly before forearm I/R (HE-EPC) or 24 h before I/R (He-LPC). Treatment of IPC was administered directly before forearm I/R. Controls = control group without ischemia; He-EPC = helium early preconditioning group; He-LPC = helium late preconditioning group; I/R = ischemia–reperfusion group; IPC = ischemic preconditioning group.

The group receiving L-NMMA received a dose of 0.4 mg·min^{-1}·dl^{-1} forearm tissue volume, and this dose effectively blocked nitric oxide production in previous studies.[12]

L-NMMA was started 5 min before He-EPC and was continued during helium preconditioning (35 min in total). Ninety minutes after termination of this preconditioning protocol, forearm blood flow (FBF) was restored to baseline values, indicative of normal eNOS activity before forearm I/R.

Preconditioning was induced by inflating the blood pressure cuff around the nondominant upper arm to 200 mmHg for three times 5 min interspersed with time 5 min reperfusion directly before I/R.

Assessment of Endothelial Function

Assessment of vascular function was performed using venous occlusion plethysmography (EC4; Hokanson, Inc., Bellevue, WA). After local anesthesia with lidocaine 2%, the nondominant brachial artery was cannulated under aseptic conditions using a 22-gauge needle. Bilateral FBF was measured with mercury-in-silastic strain gauges and expressed in ml min^{-1} 100 ml^{-1} forearm tissue volume. Forearm tissue was measured by water displacement. Endothelial function was assessed as

described before[13] and was measured in response to intraarterial infusion of the endothelium-dependent vasodilator acetylcholine (0.1, 0.5, 1.5, 5.0 µg/100 ml forearm tissue volume/min, Novartis AG, Stein, Switzerland) and the endothelium-independent vasodilator sodium nitroprusside (6, 60, 180, 600 ng/100 ml/forearm tissue volume/min, Apotheek Haagse Ziekenhuizen, The Hague, The Netherlands). Each dose was given for 5 min, and intrabrachial infusion was kept constant at a rate of 90 ml/h. To reconfirm that our postischemic measurement was consistent, we repeated our postischemic baseline measurement twice within 10 min, after which we continued the measurement with infusion of acetylcholine and sodium nitroprusside.

Blood Samples

A venous cannula was inserted in the nonischemic arm to collect blood samples at baseline, after 10 min of reperfusion[14] and after 3 h of reperfusion (at the end of the protocol) to allow activation of interleukins. Samples were centrifuged (1550g, 20 min, 20°C) within 15 min, and aliquots were snap frozen in liquid nitrogen and stored at –80°C.

Flow Cytometry of Microparticles

Samples of frozen citrate plasma of subjects from control, I/R-, and He-EPC group were analyzed for circulating cell-derived microparticles.[15] Samples were thawed, and microparticles were isolated and incubated with annexin V and the cell-specific monoclonal antibody or isotype-matched control antibodies and were analyzed for 1 min in an automated cell sorter (FACSCalibur flow cytometer with CELLQuest 3.1 software; BD Immunocytometry Systems, San José, CA; for details see Supplemental Digital Content 1, http://links.lww.com/ALN/A890).

Enzyme-linked Immunosorbent Assay

We used serum samples to determine levels of circulating interleukin (IL)-1β, E-selectin, soluble vascular cell adhesion molecule-1, and soluble intercellular adhesion molecule-1. Citrate plasma was used to determine levels of IL-6 and IL-8 (all kits from R&D systems, Minneapolis, MN). Samples were analyzed by enzyme-linked immunosorbent assay according to recommendations of the manufacturer.

Calculation and Statistics

All plethysmography results are presented as mean ± SEM. Demographic and cytokine data are presented as mean ± SD, and microparticle data are presented as median and 25–75 percentiles.

As in previous studies,[3] we planned to only perform a within-group analysis. Therefore, we performed a repeated measures ANOVA in each group and compared the first measurements (responses to acetylcholine and sodium nitroprusside) with the respective postischemic measurements after I/R. We focused on a group main effect and did not perform *post hoc* tests for each dosage. A value of $P < 0.05$ was considered significant. FBF measurements were analyzed using SPSS (version 16.0, Chicago, IL). The mean ratio of flow in the infused (measurement) arm/noninfused (control) arm was calculated (FBF measurement/control arm ratio). Baseline FBF measurement/control arm ratios were normally distributed, and comparison within groups of first and second measurement was performed by two-sided paired Student *t* test (one outlier [more than 2 SD] in the IPC group was excluded before analysis). Enzyme-linked immunosorbent assay data were analyzed by one-way ANOVA using Dunnett multiple comparison as *post hoc* test. Microparticle data were unequally distributed, and the Friedman test was used for within-group analysis.

Results

Demographic data of randomized volunteers are presented in table 1, and no statistical difference was observed between groups. All subjects tolerated the procedures without complications. Data of one patient were excluded because of violation of the preconditioning protocol (IPC group n = 9, all other groups n = 10). Helium administration was well tolerated, and no effects on blood pressure and heart rate were observed.
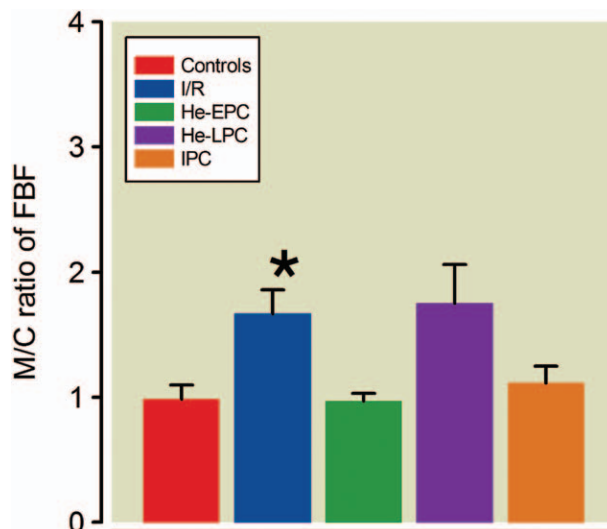

Fig. 2. Baseline FBF measurement/control arm ratio of second measurement. Bar plot showing baseline FBF measurement/control arm ratio (mean ± SD) calculated by dividing FBF from the infused (measurement) arm/noninfused (control) arm. I/R caused significant postischemic hyperemia (*$P < 0.05$) compared to the first measurement, which was prevented by He-EPC, IPC, and He-LPC. Controls = control group without ischemia; FBF = forearm blood flow; He-EPC = helium early preconditioning group; He-LPC = helium late preconditioning group; I/R = ischemia–reperfusion group; IPC = ischemic preconditioning group.

Effect of Forearm I/R on Endothelial Function

Forearm I/R resulted in a persistent postischemic hyperemia, resulting in a significant increased baseline FBF at the start of the second measurement. The prolonged postischemic hyperemia was completely abolished after IPC and HE-EPC, resulting in a similar baseline FBF at the start of the second measurement. However, after helium late preconditioning a nonsignificantly increased postischemic baseline FBF was observed (fig. 2).

Acetylcholine caused dose-dependent increases in FBF in all groups. In the control group, both the baseline and the second measurement showed similar responses to acetylcholine, illustrating the reproducibility of our methodology (fig. 3A). Forearm I/R significantly blunted the dose-dependent response to acetylcholine, reflecting postischemic endothelial dysfunction ($P = 0.001$, fig. 3B). The response to sodium nitroprusside remained unaffected after forearm I/R, and maximal increase of FBF was in the same range as maximal increase in response to acetylcholine (Supplemental Digital Content 2, fig. 1, http://links.lww.com/ALN/A891).

Effect of Helium Preconditioning on Endothelial Function

He-EPC prevented postischemic endothelial dysfunction by preserving the response to acetylcholine ($P = 0.581$ first *vs.* second measurement, fig. 3C). Even when administered 24 h before forearm I/R, helium late preconditioning preserved
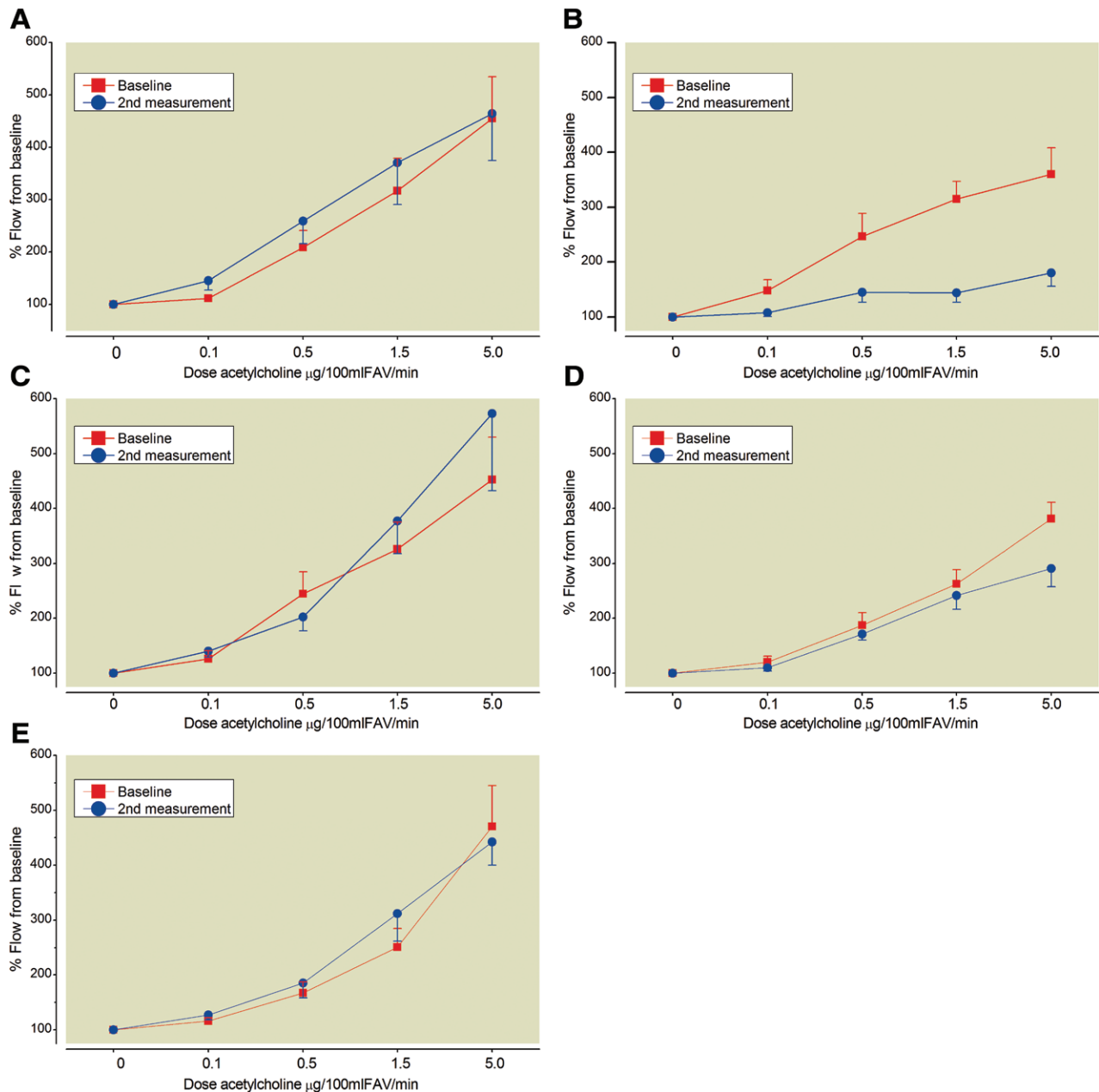
Fig. 3. Acetylcholine dose–response curves. All data are represented as mean ± SEM. *A*, Similar dose–response curves to acetylcholine for the first and the second measurement in controls. (*P* = 0.59). *B*, I/R of the forearm significantly blunted dose-dependent response to acetylcholine (*P* = 0.001), indicating postischemic endothelial dysfunction. Helium early (*C*, *P* = 0.581) and late preconditioning (*D*, *P* = 0.165) prevented postischemic endothelial dysfunction, as did ischemic preconditioning (*E*, *P* = 0.657). controls = control group without ischemia; FAV = forearm volume; He-EPC = helium early preconditioning group; He-LPC = helium late preconditioning group; I/R = ischemia-reperfusion group; IPC = Ischemic preconditioning group.

postischemic endothelial function (*P* = 0.165, fig. 3D). The protection by helium preconditioning was comparable to protection elicited by IPC (*P* = 0.657, fig. 3E).

In the additional L-NMMA group (male/female 1/7, age 23 ± 5 yr, body mass index 21.7 ± 2.1, systolic and diastolic blood pressure 122 ± 19 mm Hg and 69 ± 9 mm Hg, respectively), L-NMMA significantly reduced FBF by 61 ± 8%, indicating eNOS blockade during application of He-EPC.

This blockade, however, did not block the protective effect of He-EPC as the response to acetylcholine was preserved (*P* = 0.720, fig. 4).

Effect of I/R on Circulating Proinflammatory Cytokines and Adhesion Molecules

We measured the levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1,
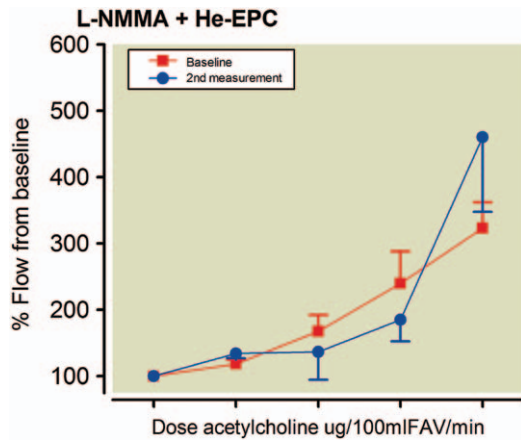
Fig. 4. Acetylcholine dose–response curves after infusion of L-NMMA during He-EPC. Infusion of L-NMMA during He-EPC did not block helium preconditioning, as postischemic endothelial function was still preserved. FAV = forearm volume; He-EPC = helium preconditioning; L-NMMA = N^G-monomethyl-L-arginine.

and E-selectin. All baseline values were within the normal limits reported for healthy subjects. To investigate whether forearm I/R would affect these adhesion molecules, we measured the levels after 10 min of reperfusion and after 3 h of reperfusion. We observed no significant effects on the plasma levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, and E-selectin.

The baseline levels of the proinflammatory cytokines IL-1β and IL-8 were all below the lowest standard provided by the manufacturer. The levels of IL-1β after 10 min and 3 h of reperfusion remained below the detection limit of the assay, and no increases were observed. Forearm I/R did not affect systemic levels of IL-8 after 10 min or 3 h of reperfusion. In contrast, IL-6 increased in all groups in time without significant differences among controls, I/R, or preconditioning groups (table 2).

Effect of I/R on Circulating Cell-derived Microparticles

To investigate whether helium preconditioning or forearm I/R affected the release of endothelial microparticles, plasma samples from the control, He-EPC, and I/R group were analyzed for the presence of microparticles (all groups n = 8). An example of the microparticle results is given in Supplemental Digital Content 2, fig. 2, http://links.lww.com/ALN/A891). Neither forearm I/R nor He-EPC affected the total levels of circulating microparticles in blood.

In line with earlier studies, we found that the levels of microparticles originating from endothelial cells, that is, microparticles binding antibodies directed against E-selectin (CD62e), VE-cadherin (CD144), or melanoma cell adhesion molecule (CD146), were very low and almost below the detection limit, except for samples from one volunteer in the I/R group who showed a strong increase in endothelial microparticles after I/R.

We further investigated the cellular origin of microparticles from platelets (CD61, CD62p, CD63), lymphocytes (CD4, CD8, CD20), monocytes (CD14), granulocytes (CD66b), or erythrocytes (CD235a). We found a large variation in the baseline levels of erythrocyte-derived microparticles in all groups, reflecting large heterogeneity in our volunteers, which is possibly caused by mild hemolysis (table 3). Forearm I/R resulted in a nonsignificant increase in systemic circulating erythrocyte–derived microparticles (table 3). Furthermore, we observed no effect of He-EPC or forearm I/R on microparticles exposing tissue factor or microparticles derived from leukocytes. Because we focused on a group main effect of endothelial function and did not perform a power analysis for the effect of forearm I/R or He-EPC on microparticles, we cannot conclude that forearm I/R affects levels of systemic circulating microparticles and that microparticles do not play a role in He-EPC on a systemic level. Because we did not investigate the levels of microparticles in the ischemic arm, we cannot exclude a possible effect on a local level.

Table 2. Adhesion Molecule Expression and Cytokine Levels at 3 h of Reperfusion

Target	Control	I/R	He- EPC	He-LPC	IPC
		3 h Reperfusion			
sVCAM-1, ng/ml	730 ± 83	716 ± 130	674 ± 246	604 ± 136	690 ± 128
sICAM-1, ng/ml	235 ± 77	213 ± 48	211 ± 77	203 ± 23	208 ± 57
E-selectin, ng/ml	37.7 ± 19.1	28.2 ± 12.1	30.4 ± 8.6	23.2 ± 7.6	32.0 ± 12.5
IL-1β, pg/ml	2.0 ± 0.7	1.3 ± 0.8	1.0 ± 0.9	0.9 ± 1.1	0.9 ± 1.0
IL-6, pg/ml	3.46 ± 2.3	4.96 ± 3.4	4.80 ± 4.1	4.43 ± 2.5	3.76 ± 2.4
IL-8, pg/ml	3.85 ± 1.7	5.07 ± 3.4	5.79 ± 2.4	3.70 ± 1.5	3.33 ± 2.5

Data are expressed as means ±SD. No statistical differences were observed between groups.

Controls = control group without ischemia; He-EPC = helium early preconditioning group; He-LPC = helium late preconditioning group; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-8 = interleukin-8, IPC = Ischemic preconditioning group; I/R = ischemia–reperfusion group; sICAM-1 = soluble intercellular adhesion molecule-1, sVCAM-1 = soluble vascular cell adhesion molecule-1.

Discussion

In this study, we show for the first time that inhalation of helium in humans prevents impairment of acetylcholine-induced vasodilation after I/R. A similar protection was observed 24 h after helium administration. Therefore, our data show that helium induces not only early but also late endothelial preconditioning in humans *in vivo*. This conclusion is supported by the improved postischemic FBF upon infusion of the endothelial-dependent vasodilator acetylcholine, whereas the response to the endothelial-independent vasodilator sodium nitroprusside was unaffected.

Forearm Blood Flow Model

A time-control group was included in our study to demonstrate that the results were reproducible. In accordance with previous studies using the same model,[3,16] ischemic preconditioning protected against endothelial dysfunction. Although an increased baseline FBF after ischemia (hyperemia) in the I/R group might interfere with postischemic response curve to acetylcholine, it is unlikely that this effect is responsible for the absent response to acetylcholine. The postischemic response to sodium nitroprusside (Supplemental Digital Content 2, fig. 1, http://links.lww.com/ALN/A891) demonstrates that vasodilation can still be achieved after I/R, indicating postischemic vasodilation was not maximal.

Acetylcholine results in calcium-mediated activation of eNOS *via* the endothelial muscarinergic receptor.[17] Under physiologic conditions, nitric oxide diffuses to the vascular smooth muscle cell layer and activates soluble guanylate cyclase, eventually leading to cyclic guanosine monophosphate–mediated vasodilation and flow increase.[18] After I/R, acetylcholine-induced vasodilation is reduced probably by a decrease in the release of nitric oxide, which can be due to eNOS uncoupling, *i.e.*, endothelial depletion of essential cofactors of eNOS such as tetrahydrobiopterin and L-arginine.[19] As a result of this uncoupling, scavenging of endothelial nitric oxide by increased reactive oxygen species can lead to production of peroxynitrite, which in turn can induce cellular injury and vasoconstriction.[20] There could be a contribution of vascular smooth muscle cell dysfunction; however, this is unlikely because sodium nitroprusside–induced vasodilation was unaltered by I/R.

Preconditioning by Inhalational Substances

Volatile anesthetics, as well as the anesthetic noble gas xenon, induce organ protection by preconditioning,[21,22] as was shown in several *in vitro*[23] and *in vivo*[8] models. This organ protective effect cannot be attributed to analgesic actions of these gases as the analgesic gas nitrous oxide did not precondition the rat heart *in vivo*.[24] Noble gases without anesthetic properties induced preconditioning in rabbits *in vivo*,[25] and for the noble gas helium both the early and late phase of preconditioning have been demonstrated.[8,26,27]

For volatile anesthetics, the translation to clinical I/R situations was made by showing the preconditioning effect of sevoflurane in patients undergoing coronary artery bypass graft surgery.[28,29] Although previous studies demonstrated a late phase of preconditioning by gases,[8,22] the present study shows for the first time that inhalation of helium induces early and also late endothelial preconditioning in humans.

In contrast to our data showing helium preconditioning in the human endothelium, another study investigating helium preconditioning in human endothelium found that helium provided modest antiinflammatory effects, but did not protect against I/R.[14] There are several differences between the two studies. First, postischemic reactive hyperemia was used to assess endothelial function, which is less reliable to measure endothelial function compared to infusion of a vasodilator like acetylcholine. Second, the lack of a distinct preconditioning protocol could be the reason why helium failed to induce preconditioning, because the noble gas was applied continuously before, during, and after ischemia. Previous clinical studies demonstrated that the preconditioning protocol plays a major role in volatile-anesthetic–induced organ protection in humans: protection could only be evoked by a distinct and repetitive stimulus.[28,29] Another difference between our study and the study from Luchinetti *et al.*[8] is the helium concentration used to induce endothelial protection (50% compared to 79%). The minimal required concentration of helium to induce preconditioning in humans is unknown and could be above 50%, although in experimental studies in rats a concentration of 30–70% helium was sufficient to induce late preconditioning, whereas 10% was not.

Possible Mechanisms of Protection

Although the mechanisms of helium-induced preconditioning are not fully clarified, some mediators have been identified and are discussed in a recent review about the possible effects of helium in different organs.[30] One experimental study showed that administration of the nonselective nitric oxide synthase inhibitor *N*-nitro-*L*-arginine methyl ester during helium preconditioning abolished cardioprotection in rabbits.[31] Data from this study suggest that helium preconditioning is mediated by nitric oxide generated by eNOS *in vivo*. To investigate the possible role of eNOS in He-EPC, we administered L-NMMA during helium breathing. Our data (see fig. 4 and Supplemental Digital Content 2, fig. 3, http://links.lww.com/ALN/A891) demonstrate that in our experiments eNOS blockade administered during helium preconditioning does not abolish endothelial protection. There are some limitations to our L-NMMA administration protocol: we only administered L-NMMA during helium preconditioning and stopped infusion before the start of I/R. We cannot rule out from the current data that a prolonged infusion of L-NMMA might be able to block the helium preconditioning effect. We carefully considered our administration protocol for L-NMMA, and administration during

Table 3. Analysis of Circulating Microparticles at Different Time Points

Marker	Controls		
	Baseline	Rep 10 min	Rep 3 h
CD235a	6.3 (4.2–20.5)	3.5 (1.96–10.0)	2.6 (2.0–9.3)
CD61	36.6 (15.7–13.8)	40.0 (10.0–50.0)	38.9 (13.4–192)
CD62p	3.0 (1.0–8.0)	2.0 (0.8–3.0)	2.4 (1.3–8.3)
Tissue factor	1.0 (0.5–3.0)	0.4 (0.2–1.0)	1.0 (0.3–2.0)
% CD62p pos. platelets	9.8 (2.9–14.6)	3.7 (2.2–13.8)	4.8 (3.8–13.0)

All data are expressed as mean ± SD. All values are corrected for isotope controls and represent ×10⁵ microparticles/ml. The percentage of positive CD62p particles was calculated by dividing particles positive for both CD62p and CD61 by the total amount of CD61-positive particles. Data are presented as median with 25–75 quartiles. No significant differences were observed between groups.

Controls = control group without ischemia; He-EPC = helium early preconditioning group; I/R = ischemia–reperfusion group.

forearm ischemia and reperfusion could have altered the postischemic damage. This is in line with another study in which infusion of L-NMMA was continued during acetylcholine infusion, resulting in a decreased response to acetylcholine even in the control group.[32] Experimental data have shown that L-NMMA, given during ischemia and reperfusion, attenuated postischemic endothelial dysfunction in the Langendorff-perfused heart,[33] making it impossible to compare results of these groups with groups not receiving L-NMMA.

Although it is very unlikely, L-NMMA might have induced preconditioning by itself, thereby overcoming a blockade of the helium preconditioning effect. However, this preconditioning effect of L-NMMA has never been demonstrated in animal studies before. Although blood flow was significantly reduced by L-NMMA, we did not observe any aspects of forearm ischemia caused by L-NMMA in the physically nonactive study situation.

Endothelial injury may lead to the increased expression of inflammatory cytokines and adhesion molecules, resulting in increased adhesion and migration of leukocytes. One of the cytokines that mediates endothelial dysfunction is tumor necrosis factor-α,[34] which stimulates the production of IL-6. IL-6 is of vital importance to induce ischemic late preconditioning,[35] and increased levels of IL-6 are associated with poor prognosis in patients with heart failure.[36]

Although we did not perform a power analysis of the effect of forearm I/R on cytokines, the current data suggest that forearm I/R does not affect the systemic levels of IL-1β, IL-6, IL-8, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, and E-selectin after 15 min or 3 h of reperfusion. However, we cannot exclude a local contribution of these cytokines to the endothelial dysfunction after I/R.

Another mechanism of endothelial dysfunction is the presence of circulating endothelial microparticles, which proved to be an independent risk factor for impaired endothelial vasodilation.[37,38] Microparticles from patients with acute myocardial infarction selectively impaired nitric oxide production and caused severe endothelial dysfunction shown by impairment of acetylcholine-induced vasodilator response in isolated vessels.[11] We showed that forearm I/R did not significantly affect the amount of circulating microparticles derived from platelets and erythrocytes. Microparticles derived from endothelial cells and leukocytes were at or below the detection limit, also after I/R. It is unknown whether forearm I/R causes microparticle release, and it is possible that a local increase of microparticles, possibly endothelial microparticles, in the venous outflow tract was missed because of systemic dilution.

Study Limitations

Because we collected our blood samples from the control arm, only systemic effects of cytokines and microparticles could be investigated. There are no previous studies in healthy volunteers that investigated cytokine release or its time course after forearm I/R. We therefore cannot exclude the possibility that there is cytokine involvement at other time points than those investigated in our present study.

In this study, we focused on group main effects of forearm I/R and helium inhalation on endothelial function, and statistical analyses between groups were not performed. We also cannot exclude effects of helium on cytokines and microparticles in a larger study population. However, previous studies found significant differences in similar sized study populations.[38]

Inhalation of helium has been shown to affect ventilation parameters in patients with chronic obstructive airway disease.[39] Volunteers inhaled helium *via* a noninvasive ventilation machine, and changes in ventilation parameters (*e.g.*, breathing frequency) were not observed. We did not measure arterial partial pressure of oxygen during helium inhalation. However, significant changes in oxygen tension after inhalation of Heliox containing 21% oxygen are not to be expected in healthy volunteers. In addition, helium inhalation was stopped at least 5 min before forearm I/R, and because helium rapidly diffuses, a significant effect on oxygen tension during forearm I/R is most unlikely. We did not investigate the direct effect of helium on FBF without I/R.

Helium is a nonanesthetic, nontoxic gas without any hemodynamic side effects that can easily be applied to patients. This inhalational gas could be a perfect instrument

I/R			He-EPC		
Baseline	Rep 10 min	Rep 3 h	Baseline	Rep 10 min	Rep 3 h
6.0 (1.0–10.0)	3.0 (2.0–20.0)	20 (4.0–50)	9.0 (4.0–36.7)	10.5 (5.1–34.4)	7.1 (3.6–3.4)
26.7 (10.0–76.2)	20.6 (9.0–44.2)	18.8 (7.2–38.5)	44.5 (33.7–52.5)	30.6 (5.1–58.9)	18.6 (5.3–35.7)
1.0 (0.4–3.0)	0.7 (0.4–1.0)	2.0 (0.7–3.0)	0.9 (0.3–4.0)	0.4 (0.3–3.9)	0.7 (0.1–1.0)
0.3 (0.1–0.5)	0.2 (0.1–0.7)	0.4 (0.3–1.0)	0.4 (0.2–0.9)	0.3 (0.2–0.5)	0.2 (0.09–0.4)
3.9 (2.0–5.7)	3.4 (2.3–6.7)	5.3 (2.5–6.9)	2.2 (1.2–6.9)	3.5 (1.8–4.7)	0.8 (1.1–9.6)

to induce preconditioning in patients subjected to clinical I/R situations, *i.e.*, coronary artery bypass graft surgery. However, whether helium preconditioning can protect patients with comorbidities such as atherosclerosis, hypertension, or diabetes mellitus still has to be investigated.

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