Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans

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1Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 2Department of Pharmacology-Toxicology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 3Department of General Internal Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 4Department of Epidemiology, Biostatistics and Health Technology Assessment, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; and 5Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom

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van den Munckhof I, Riksen N, Seeger JP, Schreuder TH, Borm GF, Eijsvogels TM, Hopman MT, Rongen GA, Thijssen DH. Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. Am J Physiol Heart Circ Physiol 304: H1727–H1732, 2013. First published April 19, 2013; doi:10.1152/ajpheart.00054.2013.—Reperfusion is mandatory after ischemia but also triggers ischemia-reperfusion (I/R) injury. Ischemic preconditioning (IPC) can limit endothelial I/R injury. Nonetheless, translation of IPC to the clinical arena is often disappointing. Since application of IPC typically relates to older patients, efficacy of IPC may be attenuated with aging. Our objective was to examine the impact of advanced age on the ability of IPC to protect against endothelial dysfunction due to I/R injury. We included 15 healthy young (20—25 yr) and 15 older (68—77 yr) men. We examined brachial artery endothelial function using flow-mediated dilation (FMD) before and after arm I/R (induced by inflation of an upper-arm blood pressure cuff for 20 min and 15 min of reperfusion). In a randomized order, I/R was preceded by IPC or a control intervention consisting of three cycles of 5 min upper-arm cuff inflation to 220 or 20 mmHg, respectively. As a result, in young men, FMD decreased significantly after I/R (6.4 ± 2.7 to 4.4 ± 2.5%). This decrease was not present when I/R was preceded by IPC (5.9 ± 2.3 to 5.6 ± 2.5%). IPC-induced protection appeared to be significantly reduced in the elderly patients (P = 0.04). Although FMD decreased after I/R in older men (3.5 ± 1.7 to 2.5 ± 1.0%), IPC could not prevent this (3.7 ± 2.1 to 2.2 ± 1.1%). In conclusion, this study is the first to observe in humans in vivo that older age is associated with an abolished effect of IPC to protect against endothelial dysfunction after I/R in the brachial artery. This provides a possible explanation for the problematic translation of strategies that reduce I/R injury from preclinical work to the clinical arena.

cardiovascular disease; endothelial function; flow-mediated dilation; ischemia

DESPITE MAJOR ADVANCES in prevention and treatment, ischemic cardiovascular disease remain the leading cause of morbidity and mortality worldwide. Current treatment for acute ischemic events in heart, brain, or kidney is timely reperfusion of the occluded artery. However, reperfusion of the ischemic tissue itself will also induce injury, commonly referred to as ischemia-reperfusion (I/R) injury (38).

Endothelial cells are particularly sensitive to I/R. Subsequent endothelial injury and swelling due to I/R can contribute to further ischemia by impeding blood flow upon reperfusion, which has been termed the “no-reflow phenomenon” and is present in the myocardium (9) as well as the brain (3). Recent evidence supports a central role for the nitric oxide pathway in I/R-induced endothelial dysfunction, as supplementation of tetrahydrobiopterin or L-arginine protects against I/R-induced endothelial dysfunction in humans (30). The no-reflow phenomenon is associated with worse clinical outcome and increased mortality in patients undergoing percutaneous coronary intervention (9). Therefore, novel therapies to limit I/R injury are urgently needed. Ischemic preconditioning (IPC), i.e., repeated preceding short periods of ischemia (26), is the most powerful strategy to limit I/R injury also in humans in vivo (16, 17, 22). A comparable protective effect can be established with remote IPC and pharmacological preconditioning (29).

Despite the potent effects of (remote) IPC and pharmacological preconditioning in preclinical research, translation of these strategies to the clinical arena is often disappointing (13). This may relate to the inclusion of young and healthy animals in preclinical studies, whereas application of IPC in the clinical setting typically relates to older patients with comorbidities. Evaluation of the impact of aging on cardioprotection is therefore prioritized by a recent position paper (13). Animal studies suggest that the protective effects of IPC are attenuated or even abolished in aging hearts (10) or brain (14), although data are conflicting (28). Also in isolated atrial trabeculae from elderly patients, IPC has no beneficial effect on the functional recovery after simulated I/R (4). To date, no previous study examined the impact of aging on the protective effects of IPC in humans in vivo.

Therefore, in this study, we examined the impact of advanced age on the protective effect of IPC on endothelial I/R injury in humans in vivo. In line with previous animal data, we hypothesized that advanced age is associated with an abolished protective effect of IPC. Since ischemia and reperfusion cannot be studied safely in heart or brain, we examined brachial artery flow-mediated dilation (FMD; i.e., a measure of peripheral artery endothelial function) before and after ischemia (20 min) and reperfusion (15 min) and use the reduction in FMD as a well-validated marker of endothelial injury. Although this provides information about peripheral vessels rather than the coronary circulation, this model is frequently used as a surrogate end point for I/R injury (17): brachial artery FMD correlates well with endothelial function of the coronary circulation.
METHODS

Participants

We included 15 young (20–25 yr) and 15 older healthy (68–79 yr) men in this study. All subjects were free of any cardiovascular disease, diabetes mellitus, hypertension (diastolic ≥ 90 and/or systolic blood pressure ≥ 140 mmHg), and hypercholesterolemia (total cholesterol ≥ 6.5 mmol/l). We also excluded (elite) athletes (performing >10 h/wk), smokers, obese subjects (body mass index (BMI) ≥ 30 kg/m²), and those who were taking medication that interferes with our primary outcome parameters. All individuals provided informed consent to participate, and the study was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre. The study was registered at ClinicalTrials.gov (NCT01606410).

Experimental Design

Subjects attended our laboratory twice (separated by at least 7 days, with a maximum of 28 days). Brachial artery endothelial function was measured with FMD in the right arm. Brachial artery FMD was measured before and after 20 min of arm ischemia and 15 min of reperfusion. This protocol of prolonged ischemia, followed by reperfusion, is repeatedly found to result in an immediate decrease in brachial artery FMD (20, 23, 37). The transient decrease in FMD is believed to reflect I/R-induced endothelial dysfunction, a finding supported by studies that successfully prevented this decline in FMD by well-established pharmacological [i.e., statins (21) and physical interventions that protect (the heart) against I/R injury. As such, studies have frequently adopted this model to examine I/R injury in conduit arteries. The assessment of FMD before and after I/R injury is performed with or without a preceding IPC stimulus (24). IPC was performed as three cycles of 5 min occlusion of the right upper arm followed by 5 min of deflation. This IPC protocol is based on previous studies that have reported a protective effect of this stimulus in the heart or peripheral tissues (8, 17, 22, 24, 26).

Measurements

Before each experiment, participants refrained from food ingestion ≥ 6 h, caffeine and products with high levels of vitamin C intake ≥ 18 h, and from strenuous physical activity ≥ 24 h. Subjects were tested at the same time of day to prevent diurnal variation in FMD response. All measurements were performed in a temperature-controlled room (22.5°C) and using recent guidelines of FMD. (33)

Flow-mediated dilation. Subjects rested in a supine position with both arms extended and immobilized, supported at an angle of ~80° abduction from the torso. Heart rate and mean arterial pressure were determined from an automated sphygmomanometer (GE Pro 300V2, Dinamap, Tampa, FL). For the assessment of FMD, a rapid inflation/deflation pneumatic cuff was placed distal to the olecranon process to provide an ischemic stimulus distal from the brachial artery to provoke vasodilatation and subsequent shear stress. A 10-MHz (T3000, Terason, Aloka, UK) multifrequency linear array probe attached to a high-resolution ultrasound machine was used to perform imaging. The brachial artery was imaged in the distal third of the upper arm. Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. A continuous Doppler velocity assessment was obtained simultaneously, and data were collected using the lowest possible insonation angle (always <60°), which did not vary during each study (33). After a resting period of at least 15 min, 1 min of baseline recording of the arterial diameter and velocity was performed. Subsequently, the occlusion cuff was inflated to 220 mmHg for 5 min. The arterial diameter and velocity recordings were restarted at least 30 s before cuff deflation as continued for at least 3 min after deflation. Peak arterial diameter and flow and the time to reach this peak after cuff deflation were recorded.

I/R injury. The rapid inflation/deflation pneumatic cuff was positioned proximally around the upper arm to provide an occlusion for 20 min, so that the brachial artery was within the ischemic zone and was exposed to I/R. The cuff was inflated for 20 min to 220 mmHg, which was followed by 15 min of reperfusion. This method is safe and frequently used. Previous studies from various groups found this protocol to result in an immediate decrease in brachial artery FMD (20, 23, 37), which is believed to reflect I/R-induced endothelial dysfunction.

Brachial Artery Diameter and Blood Flow Analysis

Analysis of the brachial artery diameter was performed using custom-designed edge-detection and wall-tracking software, which is independent of investigator bias (36). Baseline data were calculated across the 1-min preceding cuff inflation. Following cuff deflation, peak diameter was automatically detected according to an algorithm as described in detail elsewhere (5). Within-subject reproducibility of the FMD using this semiautomated software is 6.7–10.5% (coefficient of variation) (34). Postdeflation shear rate data, derived from velocity and diameter measures, were used to calculate the area under the shear rate curve (SRauc).

Statistical Analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 16). Data are presented as means ± SD unless stated otherwise. Baseline parameters were compared by paired t-tests, whereas baseline characteristics between groups were compared using an unpaired Student’s t-test. To evaluate the effect of IPC on the impact of I/R on FMD, the differences between the FMD before and after I/R were calculated. These differences were then analyzed with a linear mixed model analysis with random factor subject and fixed factors age group, IPC, and the interaction between age group and IPC. In an additional analysis, baseline characteristics were added as covariates (i.e., BMI, blood pressure, cholesterol, and glucose). In a similar way, we also evaluated the impact of preconditioning within the age groups, using a mixed model analysis with random factor subject and fixed factor IPC. To assess potential confounding by shear rate, which differed between our samples of young and elderly volunteers, we also examined the relation between the SRauc and the change in FMD after I/R using a Pearson’s correlation coefficient. The level of statistical significance was set at 0.05.

RESULTS

Baseline characteristics are presented in Table 1. When compared to young men, older men demonstrated a higher

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Young Men</th>
<th>Older Men</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>76 ± 10</td>
<td>81 ± 9</td>
<td>0.29</td>
</tr>
<tr>
<td>Height, cm</td>
<td>180 ± 7</td>
<td>177 ± 6</td>
<td>0.29</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.4 ± 2.5</td>
<td>25.5 ± 2.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>119 ± 8</td>
<td>128 ± 9</td>
<td>0.007</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>4.4 ± 0.9</td>
<td>5.2 ± 0.8</td>
<td>0.018</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.9 ± 0.5</td>
<td>1.2 ± 0.3</td>
<td>0.052</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>2.7 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>0.005</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.5 ± 0.3</td>
<td>4.9 ± 0.4</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 15 healthy young and 15 older men.
BMI, systolic blood pressure, total cholesterol, and glucose levels, whereas no differences between young and older men were found for body mass, height, diastolic blood pressure, and HDL cholesterol. All values were within the normal range.

**I/R and IPC: Young Men**

There were no significant differences in baseline brachial artery diameter, brachial artery FMD [absolute (FMDmm) or relative change (FMD%) from baseline], time to peak, or SR$_{AUC}$ between measurement days (all $P > 0.05$). In young men, FMD% decreased significantly after I/R (Fig. 1). When I/R was preceded by IPC, this decrease in FMD was abolished (change from baseline, IPC vs. control; $P = 0.01$). Also, when FMD was presented as the absolute change in millimeters, we found that the decrease in FMDmm after I/R was abolished when preceded by IPC (change from baseline, IPC vs. control; $P = 0.03$, Table 2).

We found no impact of I/R (neither with or without IPC) on the time to peak diameter, whereas I/R was associated with an increase in baseline diameter and decrease in SR$_{AUC}$ (Table 2). The change in SR$_{AUC}$ after I/R injury was similar between the control and IPC condition, whereas a significant interaction effect was found for the change in diameter (change from baseline, IPC vs. control; $P = 0.04$). We found no significant relation between SR$_{AUC}$ and the change in FMD after I/R ($r = 0.16$, $P = 0.57$).

**I/R and IPC: Older Men**

There were no significant differences in baseline brachial artery diameter, brachial artery FMD (in mm or in %), time to peak, or SR$_{AUC}$ between measurement days (all, $P > 0.05$). At baseline, older men demonstrated a significantly lower FMD% and FMDmm compared to young men (both $P = 0.001$). Older men also demonstrated a significantly larger brachial artery resting diameter ($P = 0.02$), a longer time to peak ($P = 0.04$), and a lower SR$_{AUC}$ ($P = 0.003$).

I/R resulted in a significant decrease in brachial artery FMD in older men (Fig. 1). When I/R was preceded by IPC, however, a similar decrease in FMD was observed (between I/R and IPC interaction; $P = 0.62$). Also, when FMD was presented as the absolute change in millimeters, we found a comparable decrease in FMDmm after I/R between both conditions in older men (change from baseline, IPC vs. control; $P = 0.52$, Table 2). The primary endpoint of the study was the difference in IPC-induced protection between the young and elderly subjects. IPC-induced protection appeared to be significantly reduced in the elderly patients ($P = 0.04$). After correction for the differences in baseline characteristics (BMI, blood pressure, cholesterol, and glucose), the size of the difference in protection did not change, but the $P$ value increased to 0.07.

In addition, IPC did not alter the time to peak diameter, whereas I/R was associated with an increase in resting diameter and decrease in SR$_{AUC}$ in older men (Table 2). The changes in resting diameter and SR$_{AUC}$ were similar between both conditions (Table 2). We found no significant relation between baseline SR$_{AUC}$ and the change in FMD after I/R in the older subjects or in the pooled data set ($r = 0.09$ and $P = 0.75$, and $r = 0.24$ and $P = 0.21$, respectively), excluding relevant bias by differences in shear rate (SR$_{AUC}$).

**DISCUSSION**

The purpose of this study was to examine the impact of advanced age on the protective effect of IPC on endothelial I/R injury in humans in vivo. First, we found that I/R impairs brachial artery endothelial function, which is in agreement with various other studies. Second, I/R-induced endothelial dysfunction in young men can be prevented when preceded by three cycles of 5 min ischemia of the upper arm before the ischemic event. More importantly, we demonstrated for the first time in humans that older age is associated with an abolished effect of IPC to protect against I/R-induced endothelial dysfunction in the brachial artery.

I/R-induced endothelial dysfunction is of particular importance as this contributes to impeded blood flow upon reperfusion, commonly referred to as the “no-reflow phenomenon.” The presence of the no-reflow phenomenon in the coronary arteries is frequently reported in patients with an acute myocardial infarction. In this study, we adopted a model of I/R...
injury using prolonged ischemia of the forearm. As demonstrated in various, independent laboratories (17, 21), the model of forearm I/R stress applied in the current study results in a significant decrease in brachial artery endothelial function in young as well as in older men. This indicates that our model is valid to detect I/R-induced endothelial dysfunction. Moreover, in this similar model, IPC prevents against endothelial dysfunction after I/R in healthy, young to middle-aged volunteers (17, 22). In this study, we confirm these observations in a group of young, healthy men.

The main finding of our study is that the protective effect of IPC on brachial artery endothelial I/R injury was abolished in older men. This observation is in agreement with some previous suggestions in animals (6, 10) and a human ex vivo study on isolated atrial trabeculae (4). However, this latter study failed to correct for potential confounders, such as medication and cardiovascular risk factors. Although some report an age-related decline in efficacy of IPC (1, 19), these studies are limited by their use of end points which are not valid to assess effects of preconditioning (i.e., ST-segment elevation) (29) and the inclusion of subjects with cardiovascular disease and risk factors (1, 19). In our study we included healthy, older subjects without cardiovascular risk factors. Even statistically correcting for differences between young and older men in BMI, blood pressure, cholesterol, and glucose did not change our major outcomes. More evidence for successfully including a representative healthy older population relates to the lower brachial artery FMD but also a longer time to peak diameter and larger baseline diameter, compared with young subjects. These findings confirm previous observations in healthy older men (5). Taken together, we provide the first evidence in a group of healthy older humans that the effect of IPC to prevent I/R-induced endothelial dysfunction in the forearm is abolished.

Although it was not the purpose of our study, our proof of concept study raises the question about the potential underlying mechanism. First, the attenuated effect of IPC may relate to an elevated threshold for triggering IPC protection rather than a complete loss of IPC to be protective. Longer ischemia and/or increased episodes of I/R in the IPC protocol may be necessary in the older population for IPC to be protective, such as previously described for diabetes mellitus type 2 (18). It is demonstrated, primarily based on evidence derived from cardiac tissue, that IPC cardioprotection is regulated via different pathways, such as the nitric oxide pathway, reperfusion injury salvage kinase pathway, and AMP-activated protein kinase. These pathways converge on mitochondria and prevent opening of the mitochondrial permeability transition pore upon reperfusion and subsequent cell death (35). A recent paper comprehensively discusses the impact of aging on the signaling cascades that seem to contribute to the loss of cardioprotection by IPC in the aged heart (7). Although the mechanisms of IPC-related protection may substantially differ between cardiac and vascular sites, aging seems to result in a decreased protein expression and blunted responses of signaling molecules, such as heat shock protein 70 content, extracellular ligand (e.g., IGF-1 and IL-6), decreased levels of connexin 43, or lower expression level of sarcolemmal receptors (e.g., IGF-1 receptor, bradykinin receptors) (7). Also, blunted activation of protein kinases (e.g., ERK1/2, Akt, GSK3β, or p38) and age-related mitochondrial changes have been hypothesized to contribute to the age-related loss of cardioprotection via IPC (39). Generally, these suggestions are typically made based on: 1. the importance of these signaling cascades for cardioprotection by IPC and 2. the impact of aging on these cascades. Taken together, future studies are necessary to elucidate the underlying pathways and the potential interacting effects between pathways in humans, but also distinguish between cardiac and vascular tissue as the IPC-driven effects may differ between vessel sites.

**Limitations**

Strengths of our paper include the controlled design, inclusion of homogenous healthy groups, within-subject comparison regarding the impact of IPC, and the use of observer-independent analysis. Our model has several limitations. First, we studied the impact of I/R injury and IPC in the forearm vasculature rather than directly in the cardiac tissue. However, it should be appreciated that there is a strong correlation between brachial artery FMD and coronary endothelial function (2, 25), especially when examining similar stimuli (31,
32), and brachial artery FMD has predictive capacity for future cardiovascular events (12, 15) and the extent and severity of coronary atherosclerosis (27). Nonetheless, one should take caution when extrapolating our findings to other vascular beds. Second, we did not use an endothelium independent vasodilator, so we cannot exclude that the detrimental effect of I/R on FMD reflects a reduced sensitivity of the vascular smooth muscle cells to nitric oxide. Third, the diameter of the brachial artery did not return to baseline after I/R. Based on the inverse relation between baseline diameter and FMD (33), the increase in diameter may relate to the decrease in FMD. However, the magnitude of increase in diameter was consistent across young and older subjects. Therefore, changes in diameter unlikely explain our principal findings. Another limitation is the lower SR_{AUC} in the older cohort compared with younger men. However, young and older men showed no correlation between SR_{AUC} and the change in FMD after I/R. Moreover, the changes in SR_{AUC} after I/R (with or without IPC) were similar in both groups. Therefore, it is unlikely that a difference in SR_{AUC} per se explains our findings. Finally, cardiovascular risk factors (e.g., hyperlipidemia, diabetes, and hypertension) are likely to interfere with the endogenous cardioprotective effects of IPC in humans (11). In our study, BMI, blood pressure, cholesterol, and glucose levels were all within the normal range, and none used medication known to interfere with these variables. The somewhat higher values in the older population may relate to physiological human aging, which is associated with small, but gradual, changes in these parameters. Finally, when statistically correcting for these parameters, we found a similar effect size of IPC in young and older subjects. Therefore, the conclusions drawn based on our observations in this study are valid for a typical, healthy cohort of older men.

Clinical Relevance

Our observation of an abolished effect of IPC raises questions about the impact of aging on ischemic post- or periconditioning; i.e., alternative strategies to prevent I/R injury. It is believed that the various methods of ischemic conditioning share a final common pathway that prevents opening of the mitochondrial permeability transition pore. Although speculative, advanced age may therefore also impact upon the efficacy of ischemic peri- or postconditioning. However, this requires further research in humans and is speculative at this stage. In contrast to traditional IPC, the application of remote IPC may be clinically more relevant since the latter procedure can be applied to a remote vascular bed. Whether aging also affects remote IPC is unknown and represents a logical extension from our current findings.

The results from this study demonstrate that brachial artery endothelial I/R injury can be prevented by IPC in young healthy men but not in elderly subjects. This finding may have important implications for studies that examine the efficacy of IPC as an intervention to prevent endothelial I/R injury and limited cardiac damage in a clinical setting. IPC and alternative interventions based on IPC are currently being applied in a large number of randomized controlled trials to prevent cardiac I/R injury in patients. When compared with that in preclinical studies, application of (remote) IPC in the clinical setting is often disappointing (13). This may relate to the inclusion of healthy, young animals in preclinical studies, whereas clinical trials mostly involve elderly. Our observations, therefore, highlight the importance to first examine the effects of novel cardioprotective strategies in older animals before translation is made to humans. Also, the impact of age should be considered when examining the effect of interventions that prevent or attenuate endothelial dysfunction after I/R by including an age-matched control group (preferably a one-by-one matching) and/or including age as a potential cofactor in statistical analyses.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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