Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 48, No. 4, 2006
ISSN 0735-1097/06/$32.00
doi:10.1016/j.jacc.2006.04.083

Ischemic Preconditioning

Caffeine Prevents Protection in Two Human Models of Ischemic Preconditioning

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OBJECTIVES We studied whether caffeine impairs protection by ischemic preconditioning (IP) in humans.

BACKGROUND Ischemic preconditioning is critically dependent on adenosine receptor stimulation. We hypothesize that the adenosine receptor antagonist caffeine blocks the protective effect of IP.

METHODS In vivo ischemia-reperfusion injury was assessed in the thenar muscle by ^{99m}Tc-annexin A5 scintigraphy. Forty-two healthy volunteers performed forearm ischemic exercise. In 24 subjects, this was preceded by a stimulus for IP. In a randomized double-blinded design, the subjects received caffeine (4 mg/kg) or saline intravenously before the experiment. At reperfusion, ^{99m}Tc-annexin A5 was administered intravenously. Targeting of annexin was quantified by region-of-interest analysis, and expressed as percentage difference between experimental and contralateral hand. In vitro, we assessed recovery of contractile function of human atrial trabeculae, harvested during heart surgery, as functional end point of ischemia-reperfusion injury. Field-stimulated contraction was quantified at baseline and after simulated ischemia-reperfusion, in a paired approach with and without 5 min of IP, in the presence ($n = 13$) or absence ($n = 17$) of caffeine (10 mg/l).

RESULTS Ischemic preconditioning reduced annexin targeting in the absence of caffeine (from 13 ± 3% to 7 ± 1% at 1 h, and from 19 ± 2% to 9 ± 3% at 4 h after reperfusion, $p = 0.006$), but not after caffeine administration (targeting 11 ± 2% and 16 ± 3% at 1 and 4 h). In vitro, IP improved post-ischemic functional recovery in the control group, but not in the caffeine group (8 ± 3% vs. −8 ± 5%, $p = 0.003$).

CONCLUSIONS Caffeine abolishes IP in 2 human models at a dose equivalent to the drinking of 2 to 4 cups of coffee. (The Effect of Caffeine on Ischemic Preconditioning; http://clinicaltrials.gov/ct/show/NCT00184912?order=1; NCT00184912). (J Am Coll Cardiol 2006;48:700–7)

Brief intermittent periods of ischemia and reperfusion render the myocardium more resistant to a subsequent more prolonged period of ischemia and reperfusion. This phenomenon, which is called ischemic preconditioning (IP), is the most powerful infarct size-limiting mechanism, other than early reperfusion (1,2). In every animal species studied, IP reduced infarct size by approximately 75% (2). Also in humans, several experimental models of ischemia-reperfusion injury demonstrated protection by IP (3). Release of the endogenous nucleoside adenosine and subsequent stimulation of membrane-bound adenosine A_1 and A_3 receptors has been identified as a pivotal mechanism in IP (4–6).

Caffeine is a non-selective competitive antagonist of adenosine receptors. The plasma caffeine concentration reached after regular coffee consumption is well within the concentration range that is needed to antagonize adenosine receptors (7). Based on this knowledge, we hypothesized that caffeine attenuates the cardioprotective effects of IP at concentrations that occur in daily life. A proof of this hypothesis may have important implications for patients who are at risk for cardiac ischemia.

To address this hypothesis, we used 2 complementary experimental models of ischemia-reperfusion injury in humans. In vivo ^{99m}Tc-annexin A5 scintigraphy of thenar skeletal muscle was used to study structural injury. This model is based on the highly specific binding of annexin A5 to phosphatidylserine residues, which are exposed on the outside of cellular membranes early after an ischemic insult (8). In vitro we assessed post-ischemic recovery of contractile function of human atrial trabeculae as a functional end point of ischemia-reperfusion injury (9).

METHODS

Subjects. Forty-two healthy male volunteers participated in 2 in vivo studies. They were asked to abstain from caffeine-containing beverages for at least 24 h before the experiment. Forty-four patients awaiting cardiac surgery agreed to participate in the in vitro study. Patients with

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Manuscript received February 15, 2006; revised manuscript received April 4, 2006, accepted April 10, 2006.

Abbreviations and Acronyms
IP = ischemic preconditioning
Isch Ex = ischemic exercise

atrial arrhythmias, right ventricular failure, or patients who were treated with theophylline, oral antiarrhythmics, or sulphonylureas were excluded because these drugs may interfere with IP. Written informed consent was obtained from all participants. Both protocols were approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center.

Experimental protocols. IN VIVO STUDIES. Two randomized double-blinded studies were performed (Fig. 1). In all subjects, after cannulation of medial antecubital veins of both forearms, maximal voluntary contraction was determined in the non-dominant arm with a handgrip dynamometer (Baseline Hydrolic Hand Dynamometer, Fabrication Enterprise Inc., Irvington, New York). In order to induce ischemia-reperfusion injury in thenar skeletal muscle, an ischemic exercise (Isch Ex) protocol was applied as previously described (8). In short, the circulation to the forearm was occluded for 10 min by inflation of an upper arm cuff to 200 mm Hg, which was combined with isometric contractions of the finger flexors at 50% of maximum contraction, performed rhythmically: 5 s of contraction followed by 5 s of relaxation until exhaustion. The total duration of ischemia was 10 min (index ischemia; Isch Ex). Immediately upon reperfusion, radiolabeled annexin A5 (0.1 mg protein, 500 MBq Tc-99m) was administered intravenously into the dominant arm. Both hands were imaged simultaneously at 1 and 4 h after injection using a Gamma camera (Siemens Orbiter, Hoffman Estates, Illinois, equipped with low-energy high resolution collimators) connected to a Hermes Gold image processing system (Nuclear Diagnostics, Stockholm, Sweden) as previously described (8).

In the first study (n = 24), the Isch Ex was preceded by 10 min of ischemia only and 10 min of reperfusion (IP). Subjects were randomized in a double-blinded fashion to receive either caffeine (4 mg/kg body weight intravenously over 10 min) or saline 35 min before the preconditioning stimulus.

To differentiate between a specific effect of caffeine on IP versus an effect on ischemia-reperfusion injury itself, a second study was performed. The set up of this second study (n = 18) was similar to the first study, but now without a preconditioning stimulus. The subjects also received caffeine or saline in a randomized and double-blind fashion.

In all subjects, blood was drawn before administration of caffeine/saline and just before the ischemic episode for the determination of plasma caffeine concentration. In the second study, we used a Fin-A-Pres tonometer (Biomedical Instrumentation, Neuilly, France) to record blood pressure and heart rate before caffeine/saline infusion and afterwards (just before the Isch Ex episode).

IN VITRO STUDY. The experimental set up as described by Speechly-Dick et al. (9) was used with small modifications to allow simultaneous measurement of 2 trabeculae from 1 patient. The right atrial appendage was harvested during

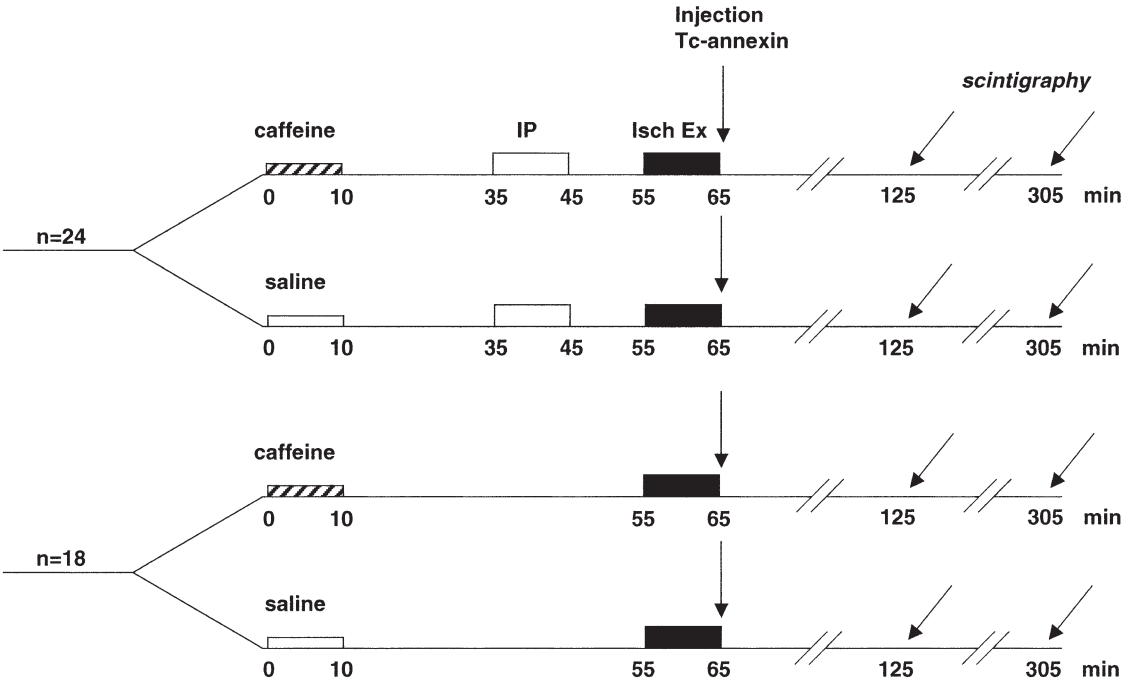

Figure 1. Schematic representation of the experimental protocol of the 2 randomized double-blinded in vivo studies. "IP" (ischemic preconditioning) indicates the 10-min period of ischemia (without concomitant exercise); "Isch Ex" (ischemic exercise) indicates the 10-min period of ischemia with isometric handgripping.

cardiac surgery before the introduction of the extracorporal circulation and immediately placed in cold (4°C) modified Tyrode's solution (NaCl 118.5 mmol/l, KCl 4.8 mmol/l, $NaHCO_3$ 13 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4$ 1.44 mmol/l, $CaCl_2$ 1.8 mmol/l, glucose 10.0 mmol/l, and pyruvate 10.0 mmol/l), which was continuously gassed with 95% oxygen and 5% CO_2. Two atrial trabeculae (diameter <1 mm; length >3 mm) were dissected, vertically suspended in an organ bath, and linked to a force transducer. Each trabecula was superfused with pre-oxygenated Tyrode's buffer (pO_2 500 to 600 mm Hg). Electrical field stimulation was performed in unstretched condition at 1 Hz using platinum ring electrodes placed on both sides of the trabeculae (pulse duration 60 ms; pulse current 40 mA). After 30 min of stimulation at unstretched conditions to allow recovery from transportation and preparation, trabeculae were gradually stretched over 15 min until maximal contractile force was achieved. After 35 min of equilibration, a baseline recording was performed during 10 min. Those trabeculae that failed to produce at least 0.2 g of developed force at the end of baseline or in which the coefficient of variation of developed force exceeded 20% were excluded (n = 14).

Immediately after baseline recordings, for each patient (n = 30) the 2 trabeculae were randomly assigned to either a stimulus for IP or continued superfusion with Tyrode's solution, so that from each patient 1 trabecula was preconditioned and the other was not. Ischemic preconditioning was induced by 5 min of simulated ischemia and 5 min of simulated reperfusion. Simulated ischemia was accomplished by superfusing the trabeculae with substrate-free modified Tyrode's solution (7.0 mM choline chloride substituted for glucose and pyruvate) and rapid pacing at 3 Hz. The superfusate was pumped into an artificial lung filled with 95% N_2/5% CO_2, which resulted in a low pO_2 of 10 to 20 mm Hg.

Subsequently, both trabeculae were subjected to 90 min of simulated ischemia and 120 min of simulated reperfusion. This protocol was performed in all patients who were included (n = 30). In the last 13 patients, caffeine was added to the superfusate at the end of equilibration at a final concentration of 10 mg/l.

Data recording and statistical analysis. All data are presented as mean ± SE.

In the in vivo protocol, all digital scintigraphic images were analyzed off-line by the same blinded investigator (W.J.G.O.), using Hermes Gold software (Nuclear Diagnostics). Region-of-interest analysis was performed for the thenar muscle region in the hand. Radioactivity was expressed as counts per pixel. To correct for background activity, the final result was expressed as the percentage difference between the experimental and control hand ("targeting").

Baseline characteristics of the 4 groups were analyzed by 1-way analysis of variance (ANOVA) followed by Scheffé's test for post-hoc comparisons (SPSS for Windows, release 12.0.1, SPSS Inc., Chicago, Illinois).

In the 2 randomized studies, differences in annexin targeting between the groups were analyzed with a repeated measures analysis of covariance (ANCOVA), with time (t = 1 and t = 4 h after injection) as within-subject factor, group (with and without caffeine pre-treatment) as between-subject factor, and workload (defined as the product of maximal voluntary force and duration of Isch Ex) as covariate (SPSS for Windows, release 12.0.1, SPSS Inc.).

For each contraction of the trabeculae, we calculated developed force (difference between maximal tension during contraction and minimal tension during relaxation), maximal speed of tension development during contraction (measure of systolic function), and maximal speed of tension reduction during relaxation (measure of diastolic function). These parameters were averaged for baseline, each subsequent 60-s period, and the last 10 min of final reperfusion. Functional recovery was expressed as a percentage of baseline. Subsequently, the effect of IP was calculated as the difference in averaged percentage recovery between the 2 trabeculae during the final 10 min of reperfusion. A paired Student *t* test was used to compare control and preconditioned trabeculae, and an unpaired Student *t* test was used to compare groups with and without caffeine (SPSS for Windows, release 12.0.1, SPSS Inc.).

Preparation of ^{99m}Tc-HYNIC-Annexin A5. Radiolabeled Annexin A5 was freshly prepared before each experiment by adding ^{99m}Tc-Pertechnetate (1,500 MBq) in the presence of stannous tricine to succinimidylhydrazinonicotinamide (HYNIC)-conjugated recombinant human Annexin A5 (NAS 2020, 0.275 mg per vial; Theseus Imaging Corp., Boston, Massachusetts). For the second study (without preconditioning), recombinant human Annexin A5 (obtained from Theseus Imaging Corp.) was conjugated with HYNIC in our own laboratory. The radiolabeling procedure of this HYNIC-conjugated Annexin A5 was identical to that of NAS 2020.

RESULTS

Baseline characteristics. In both in vivo studies, subjects were randomized to caffeine or saline administration. As such, the 2 in vivo studies comprise 4 different groups: saline-IP-Isch Ex, caffeine-IP-Isch Ex, saline-Isch Ex, and caffeine-Isch Ex. Baseline characteristics of these 4 groups are shown in Table 1. There were no significant differences between the groups, except for heart rate (Table 1). Atrial tissue was obtained in 44 patients. According to the predefined criteria, 14 patients were excluded from analysis. There were no significant differences in clinical characteristics between the groups with and without caffeine (Table 2).

In vivo studies. Plasma caffeine concentration just before ischemia was 0.2 ± 0.1 mg/l and 6.1 ± 0.4 mg/l in the first study and 0.1 ± 0.1 mg/l and 6.0 ± 0.5 mg/l in the second

Table 1. Baseline Characteristics of the Groups in the In Vivo Studies

	Saline-IP-Isch Ex	Caffeine-IP-Isch Ex	Saline-Isch Ex	Caffeine-Isch Ex
Number	12	12	9	9
Age (yrs)	25 ± 1	24 ± 1	21 ± 1	23 ± 2
Weight (kg)	76 ± 3	76 ± 3	74 ± 3	74 ± 3
Height (cm)	186 ± 2	182 ± 2	184 ± 3	183 ± 2
SBP (mm Hg)	124 ± 3	129 ± 3	126 ± 3	130 ± 3
DBP (mm Hg)	76 ± 2	76 ± 3	75 ± 2	71 ± 3
Heart rate (beats/min)	67 ± 3	71 ± 2*	61 ± 2	60 ± 3
Random glucose (mmol/l)	4.8 ± 0.2	4.4 ± 0.3		
Average coffee intake (cups/week)	11 ± 4	11 ± 3	13 ± 2	9 ± 3
Workload (kg·s)	10,000 ± 1,058	9,147 ± 594	7,219 ± 642	7,179 ± 886
Baseline plasma caffeine (mg/l)	0.3 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.1

*$p < 0.05$ versus Caffeine-Isch Ex; 1-way analysis of variance followed by Scheffé's post-hoc test.
DBP = diastolic blood pressure; IP = ischemic preconditioning; Isch Ex = ischemic exercise; SBP = systolic blood pressure.

study after saline and caffeine infusion, respectively. In the second study, we observed an increase in systolic and diastolic blood pressure after caffeine administration (Table 3).

^{99m}Tc-annexin A5 targeting in the thenar muscle 1 and 4 h after reperfusion is shown in Figure 2. When comparing the saline groups of both studies, IP reduced annexin targeting from 13 ± 3% to 7 ± 1% at 1 h, and from 19 ± 2% to 9 ± 3% at 4 h after reperfusion ($p = 0.006$, repeated measures ANCOVA). In the first randomized study, caffeine significantly reduced the effect of preconditioning: annexin targeting was 11 ± 2% and 16 ± 3% at 1 and 4 h ($p = 0.04$ vs. saline-IP-Isch Ex). In Figure 3, representative scintigraphic images of the caffeine-IP-Isch Ex and saline-IP-Isch Ex group are shown. However, in the second randomized study, caffeine did not affect annexin targeting after only the Isch Ex procedure (13 ± 3% at 1 h and 19 ± 2% at 4 h for saline-Isch Ex and 12 ± 3% and 18 ± 3% for caffeine-Isch Ex; $p = 0.8$).

Table 2. Patient Characteristics of the 2 Study Groups in the In Vitro Study

	Control Group	Caffeine Group
Number	17	13
Gender (male/female)	14/3	12/1
Age (yrs)	66.1 ± 2.3	61.5 ± 2.4
Risk factors for atherosclerosis, n (%)		
Hypertension	9 (52.9%)	7 (53.8%)
Diabetes mellitus	3 (17.6%)	2 (15.4%)
Hyperlipidemia	8 (47.1)	9 (69.2%)
Nicotine abuse	5 (29.4%)	6 (46.2%)
Indication for surgery		
CABG	12 (70.6%)	12 (92.3%)
Aortic valve replacement	5 (29.4%)	1 (7.7%)
Medication, n (%)		
Beta-blocker	12 (70.6%)	12 (92.3%)
ACE inhibitor	5 (29.4%)	6 (46.2%)
Angiotensin II receptor antagonist	2 (11.8%)	0 (0%)
Calcium-channel blocker	8 (47.1%)	3 (23.1%)
Nitrate	9 (52.9%)	8 (61.5%)
Aspirin	11 (64.7%)	12 (92.3%)
HMG CoA reductase inhibitor	12 (70.6%)	9 (69.2%)
Insulin	1 (5.9%)	2 (15.4%)
Perioperative medication, n (%)*		
Volatile anesthetics	3 (25%)	5 (46%)
Opioids	12 (100%)	11 (100%)

*This information could not be obtained in 5 subjects from the control group and 2 subjects from the caffeine group.
ACE = angiotensin-converting enzyme; CABG = coronary artery bypass grafting; HMG CoA = 3-hydroxy-3-methylglutaryl coenzyme A.

In vitro study. Baseline contractile force was similar for all groups of trabeculae (Table 4) ($p = 0.7$, one-way ANOVA). After 90 min of simulated ischemia and 120 min of reperfusion, only a partial recovery of contractile force was observed (Fig. 4). Ischemic preconditioning improved recovery from 43 ± 3% to 51 ± 4% ($p = 0.008$), from 45 ± 4% to 56 ± 7% ($p = 0.055$), and from 46 ± 4% to 56 ± 5% ($p = 0.017$) for developed force, systolic and diastolic functions, respectively.

In the presence of caffeine, IP changed recovery in contractile function from 51 ± 5% to 43 ± 3% ($p = 0.10$), from 51 ± 5% to 44 ± 3% ($p = 0.20$), and from 54 ± 5% to 48 ± 3% ($p = 0.20$) for developed force, systolic and diastolic functions, respectively. Thus, in the presence of caffeine, IP did no longer improve recovery of function. If anything, a tendency to the contrary was observed. The effect of IP on recovery of contractile force differed significantly between the control group and the caffeine group ($p = 0.003$).

DISCUSSION

Caffeine is one of the most widely consumed pharmacologically active compounds worldwide, mainly derived from dietary sources such as coffee and tea (7). Daily intake of caffeine in U.S. consumers is estimated at 4 mg/kg per day, which is even higher in some European countries (10). In the present study, we demonstrated that administration of caffeine, in a dose comparable to this estimated daily dose, abolishes protection from IP in 2 experimental models in humans.

Traditionally, IP is defined as myocardial infarct size reduction induced by a preceding brief period of ischemia and reperfusion (1). In humans, however, this histologic end point cannot be used experimentally to study IP. Therefore, several surrogate end points of ischemia-reperfusion injury have been developed to study IP in humans in an experi-

Table 3. Hemodynamic Values at Baseline (Before Caffeine/Saline) and Just Before the Ischemic Exercise Period (After Caffeine/Saline) in the Group Without Preconditioning (the Second In Vivo Study)

Groups	DBP Before	DBP After	SBP Before	SBP After	HR Before	HR After
Caffeine (n = 9)	73 ± 2	87 ± 3*	107 ± 3	122 ± 5*†	73 ± 4	69 ± 3
Saline (n = 7)	80 ± 4	85 ± 4	113 ± 5	114 ± 4	68 ± 4	67 ± 5

*$p < 0.005$ for effect of caffeine administration; †$p < 0.05$ for comparison between effect of caffeine and effect of saline.
HR = heart rate; other abbreviations as in Table 1.

mental setting. In the present study, we used 2 different, complementary, and previously validated experimental models to study the effect of caffeine on IP in humans.

The in vivo part of our study is based on the specific and high-affinity binding of annexin A5 to phosphatidylserine residues on cellular membranes. It is well documented that loss of membrane asymmetry is an early general feature of apoptosis, resulting in externalization of phosphatidylserine residues in affected cells, thus providing binding sites for annexin A5 (11,12). By labeling recombinant annexin A5 with ^{99m}technetium, it is possible to detect these cellular changes in vivo with a gamma camera. Previously, this method has been used to visualize apoptotic cells in murine and human hearts in vivo (13,14). Concomitant use of conventional methods for detection of apoptosis revealed that, indeed, annexin A5 specifically binds to cells with apoptotic morphology and co-localizes with the presence of activated caspase-3 (15). Also, annexin A5 targeting could be diminished by pre-treatment with caspase inhibitors (13). Because IP reduces myocardial ischemic injury in part by inhibition of apoptosis (16), we used ^{99m}Tc-annexin A5 scintigraphy as a model to study IP in humans. We demonstrated previously that 10 min of ischemia of the non-dominant forearm combined with isometric handgrip exercise (Isch Ex) increases targeting of annexin A5 to the thenar muscle, expressed as difference in ^{99m}Tc activity between the experimental and control hand (8). This increased targeting could be prevented by a preconditioning stimulus of 10 min ischemia and reperfusion before Isch Ex. Similarly, pre-treatment with adenosine (administered into the brachial artery) (8) and the nucleoside uptake inhibitor dipyridamole (orally, twice daily for 1 week) (17) reduced annexin targeting.

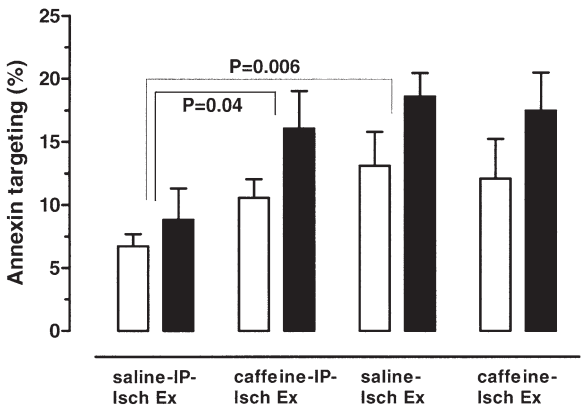

Figure 2. Annexin A5 targeting in the thenar muscle at 1 **(open bars)** and 4 **(solid bars)** h after reperfusion in the different groups (expressed as percent difference between experimental and control hand). The p values indicate differences between groups as assessed by repeated measures analysis of covariance with time (t = 1 and t = 4 h after reperfusion) as within-subject factor, group (as indicated on the x-axis) as between-subject factor, and workload as covariate. IP = ischemic preconditioning; Isch Ex = ischemic exercise.

In the present in vivo studies, we confirmed that, indeed, the preconditioning stimulus reduced annexin A5 targeting induced by Isch Ex. In the first randomized trial, pre-treatment with caffeine significantly reduced the effect of IP on annexin targeting. In fact, annexin targeting in preconditioned subjects pre-treated with caffeine was similar to the targeting in control subjects who were not preconditioned, indicating that the beneficial effect of IP is completely abolished. In the second randomized trial, administration of caffeine did not affect annexin targeting after Isch Ex without preconditioning, indicating that caffeine selectively inhibits the effect of preconditioning on annexin targeting.

To confirm that these effects of caffeine are also present in myocardial tissue, we performed an additional in vitro study on human atrial trabeculae, obtained during heart surgery, using recovery of contractile function as an end point of ischemia-reperfusion injury. This model has been used in several previous studies, which consistently demonstrated that preconditioning increases post-ischemic recovery of contractile function (6,9,18–20). Also, these studies showed that preconditioning in this model is dependent on stimulation of opioid receptors and protein kinase C, and opening of K_{ATP} channels (9,18), and can be mimicked by stimulation of adenosine A_1 and A_3 receptors, comparable to studies using histologic infarct size as an end point of ischemia-reperfusion injury (4,5,21–23). It has to be realized that recovery of mechanical function after ischemia is influenced by both the number of surviving myocytes and the effect of stunning of these myocytes. The effect of preconditioning on stunning is much more controversial than its effect on cellular death (2). However, because in the present model of 90 min of ischemia an effect of preconditioning has consistently been demonstrated, cellular death probably plays a major role in determining functional outcome.

In our study, the effect of IP was less than in previous reports from other groups (6,9). Interestingly, this is completely caused by the lower functional recovery of control trabeculae in these previous reports. Differences in experimental set up, patient group, and perioperative management could possibly account for this difference. For example, all patients included in the present study received opioid receptor agonists, and approximately one-third of patients

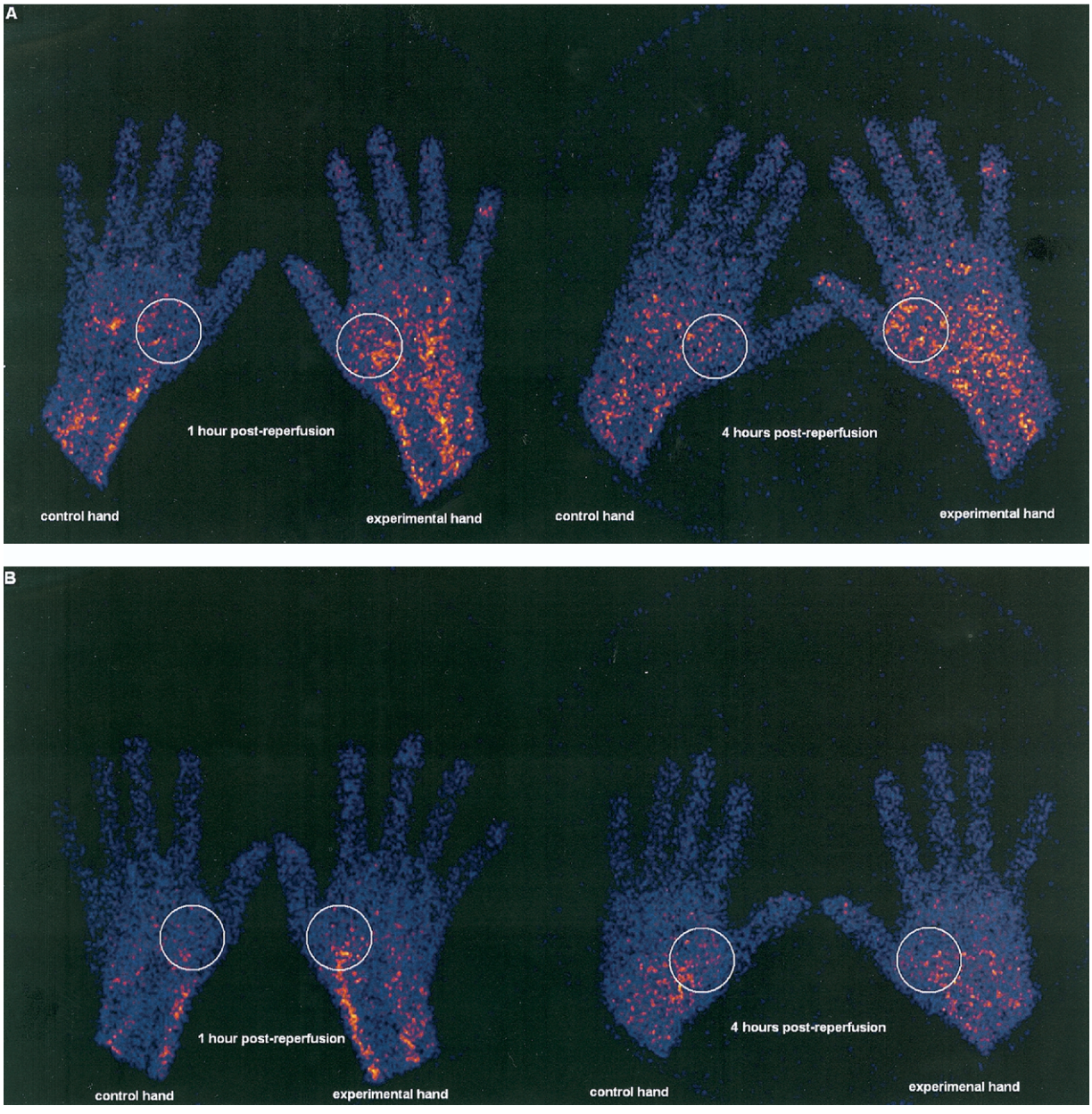

Figure 3. Representative scintigraphic images of the experimental and control hands at 1 and 4 h after reperfusion in preconditioned subjects pre-treated with caffeine **(A)** and saline **(B)**. Annexin targeting was 2% and 3% after saline administration (saline-ischemic preconditioning-ischemic exercise) and 19% and 27% after caffeine administration (caffeine-ischemic preconditioning-ischemic exercise) at 1 and 4 h, respectively.

received volatile anesthetics before resection of the atrial tissue. Because these drugs mimic IP (24), this may have resulted in improved functional recovery in the control trabeculae compared with that in previous studies, leaving less space for additional protection by IP. Unfortunately, in these previous studies, perioperative medication use was not mentioned. In the present study, post-ischemic recovery of contractile function was significantly increased by preconditioning in the saline group. In contrast, in the caffeine group, IP did not potentiate recovery of contractile function. If anything, a reduction of functional recovery was observed in the preconditioned trabeculae in the presence of caffeine. Interestingly, recovery of contractile force in the non-preconditioned trabeculae was higher in the caffeine group, although this was not significant ($p = 0.2$). Probably, this reflects a transient positive inotropic effect of caffeine, which is observed as long as caffeine is present. Indeed, in the

Table 4. Baseline Contractile Force of the Different Groups of Trabeculae (Mean ± SE)

Groups	Baseline Force (g)
Control, IP	0.50 ± 0.05
Control, no IP	0.47 ± 0.05
Caffeine, IP	0.57 ± 0.06
Caffeine, no IP	0.52 ± 0.05

IP = ischemic preconditioning.

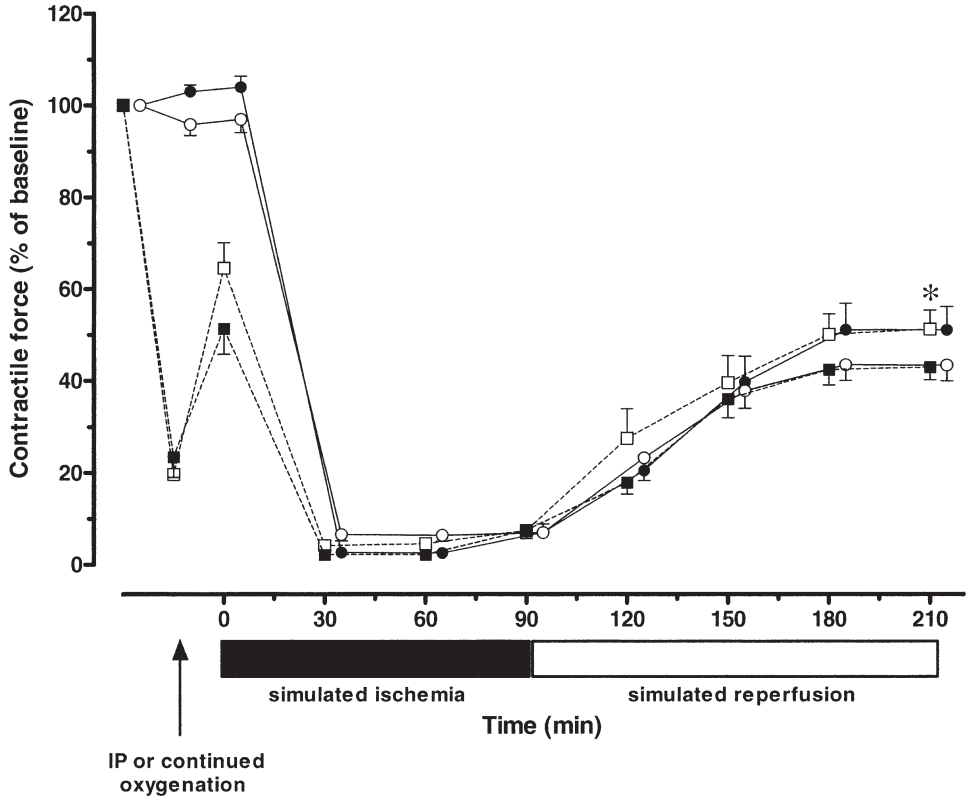

Figure 4. The course of contractile force (expressed as percentage of baseline force) in time in control trabeculae (n = 17) with **(open squares)** and without **(open circles)** preconditioning and in caffeine pre-treated trabeculae (n = 13) with **(filled squares)** and without **(filled circles)** preconditioning. *p = 0.008 for the effect of preconditioning on post-ischemic recovery of contractile force in the saline group. For the sake of clarity, the symbols representing the non-preconditioned groups are slight shifted to the right. IP = ischemic preconditioning.

subset of trabeculae without preconditioning, we observed a steady increase in contractile force in the 20 min immediately after administration of caffeine as compared with that in the control trabeculae (data not shown). This is in concordance with previous studies demonstrating positive inotropic effects of xanthine derivatives (25,26). This could be caused by antagonism of the negative inotropic effect of endogenous adenosine, which is continuously produced by cardiomyocytes, although it is suggested that alternative mechanisms might also be involved (27). However, inhibition of phosphodiesterase and mobilization of intracellular calcium is induced only at higher caffeine concentrations than the concentration achieved in our study (7). This positive inotropic effect of caffeine is also present in the preconditioned trabeculae, so any beneficial effect of preconditioning should have increased recovery of contractile force as compared with the recovery without preconditioning.

The plasma caffeine concentration immediately before the initiation of forearm ischemia averaged approximately 6 mg/l in the in vivo studies and was slightly higher in the in vitro study. This concentration occurs in daily life after an intake of 2 to 4 cups of coffee (28,29). At this concentration, the pharmacologic actions of caffeine are solely due to binding to adenosine receptors and antagonism of the actions of endogenous adenosine. Other mechanisms of action of caffeine, such as inhibition of phosphodiesterase or calcium release from intracellular stores, occur at higher concentrations (7).

Impairment of the powerful infarct size-limiting effect of IP by caffeine would theoretically cause an association between caffeine use (especially derived from coffee consumption) and clinical end points of ischemia-reperfusion injury. However, the association between coffee intake and cardiovascular disease remains controversial, although for subjects consuming more than 5 cups of coffee daily, there seems to be an increased risk for cardiovascular disease (30,31). Interestingly, a recent study showed that among patients with a CYP1A2 genotype predicting slow caffeine metabolism, intake of coffee (even 2 to 3 cups daily) was associated with an increased risk of non-fatal myocardial infarction, suggesting that caffeine does play a role in this association (32). When considering these epidemiologic trials in the light of our present results, it has to be realized that inhibition of preconditioning will not increase the incidence of cardiovascular events per se, but rather would be expected to adversely affect the outcome after myocardial infarction (i.e., the incidence of cardiac failure or death) in the subset of patients experiencing pre-infarction ischemia. This potential effect has generally not been evaluated in studies on the association of coffee consumption and the incidence of cardiovascular disease. Also, our study refers to

the acute effects of a single dose of caffeine. This does not necessarily hold for chronic use, especially because tolerance to the pharmacologic effects of caffeine has been described (33). However, several publications have shown that a considerable fraction of the population does not develop complete tolerance to caffeine despite the daily use of caffeinated products (34,35).

In conclusion, the present study shows that caffeine, at a concentration reached in daily life after drinking 2 to 4 cups of coffee, impairs the protection afforded by IP in 2 experimental models in humans. This observation provides an experimental basis to study the effects of caffeine consumption on cardiovascular morbidity and mortality in patients who are at increased risk for ischemic events.

Acknowledgments

The authors would like to thank Petra van den Broek for the determination of caffeine concentrations. The investigators appreciate the support of the staff of the Clinical Research Center Nijmegen. The authors would also like to thank Dr. J. L. Vanderheyden, Theseus Imaging Corporation, Boston, Massachusetts, for donating the NAS 2020 kits for preparation of ^{99m}Tc-HYNIC-rh-Annexin A5.

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