Caffeinated Coffee Blunts the Myocardial Protective Effects of Statins against Ischemia–Reperfusion Injury in the Rat

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Abstract

Purpose We asked whether caffeinated coffee (CC) blunts the infarct size (IS)-limiting effects of atorvastatin (ATV).
Background Adenosine receptor activation is essential for mediating the IS-limiting effects of statins. Caffeine is a nonspecific adenosine receptor blocker, and thus drinking CC may block the myocardial protective effects of statins.
Methods Rat received 3-day ATV (10 mg/kg/day) or water by oral gavage once daily. Drinking water was replaced by water + sugar (7.5 g/100 ml), CC with sugar, or decaffeinated coffee (DC) with sugar. On the 4th day, rats were anesthetized and underwent 30 min of coronary artery occlusion and 4 h reperfusion. Area at risk was assessed by blue dye and infarct size by TTC.

Results Body weight and area at risk was comparable among groups. IS was $25.1 \pm 3.9\%$ of the area at risk in the control group. In rats not receiving ATV, CC ($25.5 \pm 3.1\%$) and DC ($34.0 \pm 2.8\%$) did not affect IS. IS was significantly reduced by ATV in the water + sugar ($11.7 \pm 0.7\%$, $p=0.015$) and DC ($11.5 \pm 1.0\%$; $p<0.001$) groups, but not in the CC group ($32.3 \pm 3.0\%$; $p=0.719$). ATV increased myocardial levels of Ser-473 phosphorylated Akt in the water + sugar and DC groups, but not in the CC group.
Conclusions CC, but not DC, abrogated the IS-limiting effects of ATV by blocking the adenosine receptors and preventing the phosphorylation of Akt. CC did not affect IS in rats not receiving ATV.

Key words infarct size · atorvastatin · adenosine · coffee · caffeine

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Introduction

Although the effects of caffeinated coffee on cardiovascular morbidity and mortality have been thoroughly studied, controversy remains as to outcomes among case control and cohort studies. Two large metanalyses suggested that consuming more than five cups per day increases the risk of coronary artery disease [1, 2]. Cornelis et al. studied 2,014 cases with first acute nonfatal myocardial infarction and compared them to 2,014 age, gender, and area of residence matched controls and reported that intake of coffee is correlated with an increased risk of myocardial infarction only among individuals with a CYP1A2 genotype associated with slow caffeine metabolism, suggesting that caffeine plays a role in the association between coffee and myocardial infarction [3]. On the other hand, Silletta et al. did not find a correlation between coffee consumption

and cardiovascular events among 11,231 Italian patients with recent myocardial infarction enrolled in the GISSI (Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto miocardico)—Prevenzione trial [4]. Caffeine was shown to abolish ischemic preconditioning in human atrial trabecula *in vitro* and in human thenar muscles *in vivo* [5]. However, the effect of oral consumption of caffeinated coffee (CC) on myocardial infarct size is not known.

Caffeine (1, 3, 7-trimethylxanthine) is a nonselective competitive antagonist of adenosine [6]. At concentrations occurring in regular daily consumption, caffeine binds with high affinity to A_1, A_{2A} and A_{2B} adenosine receptors (AR) and with lower affinity to A_3 AR [7]. Adenosine has a major role in mediating ischemic preconditioning [8–12] and postconditioning [12–14]. Adenosine stimulates Akt and extracellular signal regulated kinases (ERK 1/2) pathways [14–19], activates endothelial nitric oxide synthase (eNOS) [20–24] and promotes cell survival. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) activate ecto-5′-nucleotidase and increase myocardial tissue concentrations of adenosine [25–27]. Adenosine, generated by ecto-5′-nucleotidase, is involved in the infarct-size limiting effects of statins [26]. A nonselective AR blocker, 8-sulfophenyl theophylline (8-SPT), administered during coronary occlusion blunts the infarct limitation effects of statins in dogs [26]. Both theophylline and 8-SPT blocks atorvastatin (ATV) induction of ERK 1/2, Akt and eNOS phosphorylation in the rat heart [25]. In addition, aminophylline administered during angioplasty abolished ischemia tolerance mediated by statins in patients [28].

We assessed whether oral consumption of caffeinated and/or decaffeinated coffee has an effect on myocardial protection by ATV in the rat.

Methods

Animal care

The experimental designs and animals care were conducted in accordance with 'The Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol was approved by the UTMB IACUC. Male Sprague–Dawley rats were housed at controlled room temperature (24.5–25.0°C).

Materials

We used crushed tablets of atorvastatin (ATV) (Pfizer, US Pharmaceuticals). Caffeinated (CC) and decaffeinated (DC) coffee was purchased daily at one of the local coffee shops

at the University of Texas Medical Branch campus. Monoclonal anti-β-Actin antibodies were purchased from Sigma (St. Louis, MO). Anti-Akt antibodies and anti-Ser[473] phosphorylated-Akt antibodies were purchased from Cell Signaling (Beverly, MA). Caffeine standard (99.6% purity) was purchased from Sigma.

Drugs and pretreatment

Rats received 3-day pretreatment with: (1) water; (2) water + ATV; (3) CC; (4) CC + ATV; (5) DC; (6) DC + ATV. ATV (10 mg/kg/day) or water alone was administered by gastric gavage once daily. Water, CC and the DC were sweetened with sugar (7.5 g/100 ml) and were added daily to the drinking bottle, serving as the sole source of fluids. Body weight was monitored daily to assess for fluid intake. Sixteen hours after the last oral dose of ATV or placebo the rats were anesthetized and either subjected to surgery (infarct size protocol; $n=8$ in each group) or the hearts were harvested for immunoblotting without being subjected to regional ischemia ($n=4$ in each group).

Additional rats received CC or DC as above ($n=3$ in each group) and on the 4th day blood samples were assessed for caffeine levels by high performance liquid chromatography (HPLC). Random samples of CC and DC were also assessed for caffeine concentrations by HPLC.

Infarct size (IS) surgical protocol

The rat model of myocardial ischemia–reperfusion injury has been described in detail previously. [29–32]. On the fourth day rats were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg). The animals were intubated and connected to an animal ventilator (Harvard Apparatus, Model 683, South Natick, MA) and ventilated using FIO_2 of 30%. Rectal temperature was monitored and body temperature was maintained between 36.7°C and 37.3°C with the aid of a heating lamp and heating pad. The left carotid artery was cannulated for monitoring heart rate and blood pressure, the chest was opened and a snare was placed around the left coronary artery to produce regional ischemia. Isofluorane (1–2.5% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. The snare was released after 30 min ischemia and myocardial reperfusion was verified by change in the color of the myocardium. Subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed and the rats were recovered from anesthesia. Four hours after reperfusion the rats were re-anesthetized, the coronary artery was reoccluded, 1.5 ml of Evan's blue dye 3% was injected into the right ventricle and the rats euthanized under deep anesthesia. Heart rate and mean blood pressure were noted at baseline (10 min after

completion of surgery); just before coronary artery occlusion); at 25 min of ischemia; and at 20 min of reperfusion.

The pre-specified exclusion criteria were lack of signs of ischemia during coronary artery ligation, lack of signs of reperfusion after release of the snare, prolonged ventricular arrhythmia with hypotension, and area at risk ≤10% of the LV weight.

Determination of ischemic area at risk (IAR) and IS

Hearts were excised and the left ventricle was sliced transversely into six sections. Slices were weighed and incubated for 15 min at 37°C in 1% buffered (pH=7.4) 2,3,5-triphenyl-tetrazolium-chloride (TTC), fixed in 10% formaldehyde and photographed in order to identify the IAR (uncolored by the blue dye), the IS (unstained by TTC), and the non-ischemic zones (colored by blue dye). The area of IAR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice and the results summed to obtain the weight of the myocardial IAR and IS [29–32].

Western blot analysis

Rats were treated as above, anesthetized and the hearts were removed (without being subjected to ischemia) and rinsed with cold PBS (pH 7.4), containing 0.16 mg/ml heparin to remove red blood cells and clots. Myocardial samples from the anterior left ventricular wall were frozen rapidly in liquid nitrogen, homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and the total protein concentration was determined using the Lowry protein assay. The protein samples were subjected to SDS-PAGE with 4–20% gradient polyacrylamide gel and transferred to pure nitrocellulose membrane (0.45 μm) (Bio-Rad). After blocking with 5% skim milk in Tris-buffered saline, the membrane was incubated overnight at 4°C with primary antibodies against Akt, or Ser^{473} P-Akt and secondarily with HRP-conjugated anti-mouse or anti-rabbit antibodies. The immunoblots were developed using ECL western Blotting Detection Reagent (Amersham). The protein signals were pictured by an image-scanner and analyzed using Image J software (National Institutes of Health, Bethesda, MD). The strength of each signal was normalized to the corresponding β-actin stain signal. Data are expressed as a ratio between the protein and the corresponding β-actin signal density.

Determination of caffeine concentrations in coffee samples and in blood

Coffee samples were filtered with a 0.45 nylon filter to remove particulates. One milliliter of plasma samples was mixed with 600 mg of ammonium sulfate and shaken for 1 min. A liquid–liquid extraction was applied to the samples using 6 ml of a mixture of ethyl acetate and isopropyl alcohol (8/1; v/v). The samples were shaken for 2 min and centrifuged at 6,000×g for 20 min. Five milliliter of the organic phase was collected, dried in a Savant vacuum centrifuge and the residue was dissolved in 100 μl KH_2PO_4. The filtered coffee samples and the plasma samples were then analyzed by high performance liquid chromatography (HPLC) using a Waters C18 reversed phase 150 mm× 4.6 mm column. A binary gradient consisting of 15 mM potassium phosphate buffer, pH 4.9 and 60% Acetonitrile/ Phosphate buffer (volume/volume) was used for the separation. The flow rate was 1.2 ml min using two Beckman 126 pumps. Detector Schoeffel 770 UV–Visible UV 276 nm. Samples were injected using a Waters 717 autosampler [33]. Data was collected and analyzed using Waters Millennium software.

Statistical analyses

Data are expressed as mean ± SEM. Comparisons among the groups were performed by one-way ANOVA with Sidak correction for multiple comparisons (SPSS ver. 14.0). The differences in heart rate (HR) and mean blood pressure (MBP) were compared using two way repeated measures ANOVA with Holm–Sidak multiple comparison procedures (SigmaStat ver. 3.0.1). Values of $p<0.05$ were considered statistically significant.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

On separate days caffeine levels were 514, 292, 284, 235 and 365 μg/ml (average 338.0±48.7 μg/ml) in CC coffee, and 24, 17, 26, 25.5, 37, 22.5 and 22 μg/ml (average 24.9± 2.3 μg/ml) in DC coffee. The caffeine plasma levels were 5.80, 6.03 and 6.17 μg/ml (average 5.99±0.11 μg/ml) for rats receiving CC and 1.72, 1.74 and 1.74 μg/ml (average 1.73±0.01 μg/ml) for rats receiving DC.

Infarct size

A total of 51 rats were studied. Two rats died during the initial surgery and one was excluded due to lack of signs of ischemia during coronary artery occlusion, all from the CC group.

Body weight, left ventricular weight and the size of IAR were comparable among groups (Table 1). In contrast, IS, expressed either in milligram (Table 1) or as a percentage of the IAR (Fig. 1) was significantly smaller in the ATV-

Table 1 Body weight, left ventricular (LV) weight, ischemic area at risk (IAR) and infarct size (IS) of the rats

	Water + sugar (n=8)		CC (n=8)		DC (n=8)		p value
ATV	−	+	−	+	−	+	
Body weight (g)	301±2	302±2	3,001±2	302±3	300±2	302±1	0.977
LV (mg)	1,111±9	1,099±7	1,108±11	1,123±9	1,122±11	1,103±12	0.428
IAR (mg)	335±11	349±8	337±10	365±11	354±6	347±7	0.202
IS (mg)	86±15	41±3	85±9	118±13	120±9	40±4	<0.001

treated rats among those receiving water + sugar or DC. ATV did not limit IS among rats receiving CC. CC ($p=$ 1.00) and DC ($p=0.307$) had no significant effect on IS compared to the control group.

Heart rate

Overall, the treatment assignment had no significant effect on heart rate ($p=0.145$ for the treatment effect). There was a significant change over time ($p<0.001$). There was a statistically significant interaction between treatment and time ($p<0.001$). At baseline there were no significant difference in heart rate among groups; however, as expected before occlusion heart rate was significantly faster in the CC group than in all other groups. In contrast, during ischemia heart rate was significantly slower in the CC group than in the control group, although the absolute difference was small. At reperfusion heart rate was significantly slower in the ATV group than in the control group (Fig. 2).

Mean blood pressure

Overall, there were significant treatment ($p=0.015$) and time ($p<0.001$) effects. However, there was not a statisti-

cally significant interaction between treatment and time ($p=0.055$). Mean blood pressure decreased during ischemia and reperfusion in all groups. Mean blood pressure was significantly lower in the CC group than in the control, DC and DC + ATV groups, although the differences were small (Fig. 3).

Akt phosphorylation

ATV, CC and DC did not affect myocardial total Akt levels (Fig. 4a and b). ATV increased myocardial levels of P-Akt in the water + sugar treated rats. CC, but not DC blocked the ATV effect on Akt phosphorylation (Fig. 4a and c).

Discussion

The main findings of the present study are that CC blocked the ATV induction of Akt phosphorylation and the infarct size limiting effect of ATV, whereas DC had no such effects.

Body weights of the rats in the various groups were comparable at the beginning of the protocol and before surgery, suggesting that the rats drank the coffee and were not dehydrated.
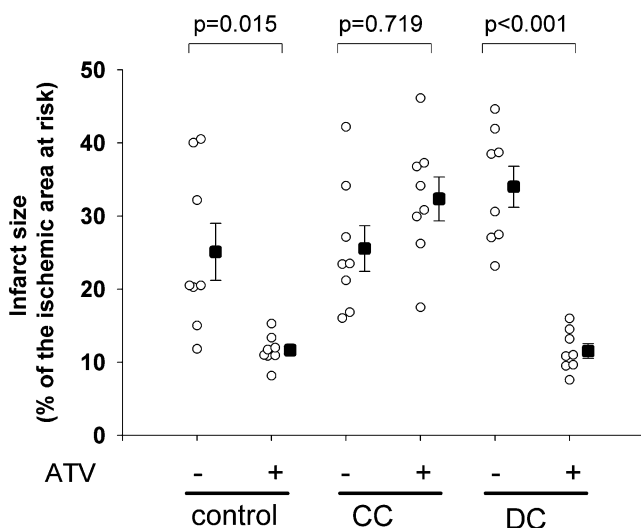

Fig. 1 Myocardial IS (% of the IAR) in the rats receiving water + sugar (control), caffeinated coffee (CC) or decaffeinated coffee (DC) with or without ATV
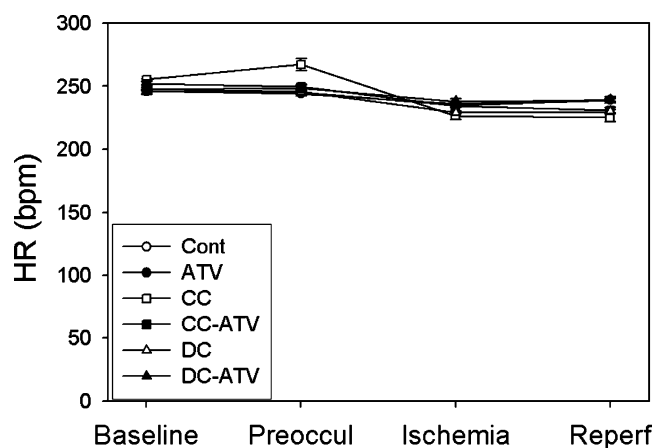

Fig. 2 Mean heart rate (bpm) at baseline, before coronary artery occlusion, 25 min of ischemia and 20 min of reperfusion. Overall, the treatment assignment had no significant effect on heart rate ($p=0.145$ for the treatment effect). There was a significant time effect ($p<0.001$). There was a statistically significant interaction between treatment and time ($p<0.001$)
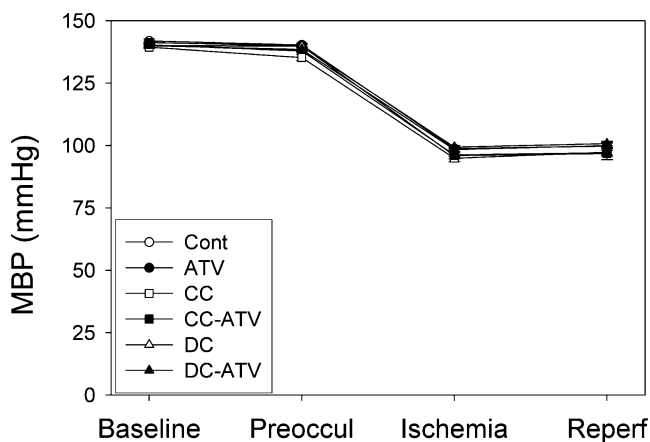
Fig. 3 Mean blood pressure (MBP) (mmHg) at baseline, before coronary artery occlusion, 25 min of ischemia and 20 min of reperfusion. Overall, there were significant treatment ($p=0.015$) and time ($p<0.001$) effects. However, there was not a statistically significant interaction between treatment and time ($p=0.055$)

As expected, heart rate before occlusion was faster in the CC group. Interestingly, despite the fact that coffee and caffeine are associated with hypertension, mean blood pressure was significantly lower in the CC group than in the control, DC and DC+ATV groups, although the differences were small.

Caffeine (1, 3, 7-trimethylxanthine) is a nonselective competitive antagonist of adenosine [6]. The caffeine content of coffee varies depending on the type of coffee and brewing method [34–38]. For example, it has been reported that a 16-oz cup of regular Starbucks coffee contains 250–564 mg of caffeine (528 to 1,382 µg/ml) with wide day to day variations. [35, 36] We found much lower concentrations (338.0 ± 48.7 µg/ml). In contrast, it has been reported that DC contains less than 17.7 mg per 16-oz cup (<37 µg/ml) [36, 37]. It has been reported that the Starbucks brewed DC contains 12.0–13.4 mg per 16-oz cup (25 to 28 µg/ml) [39]. Our findings (24.9 ± 2.3 µg/ml) are within the reported range. Caffeine is completely
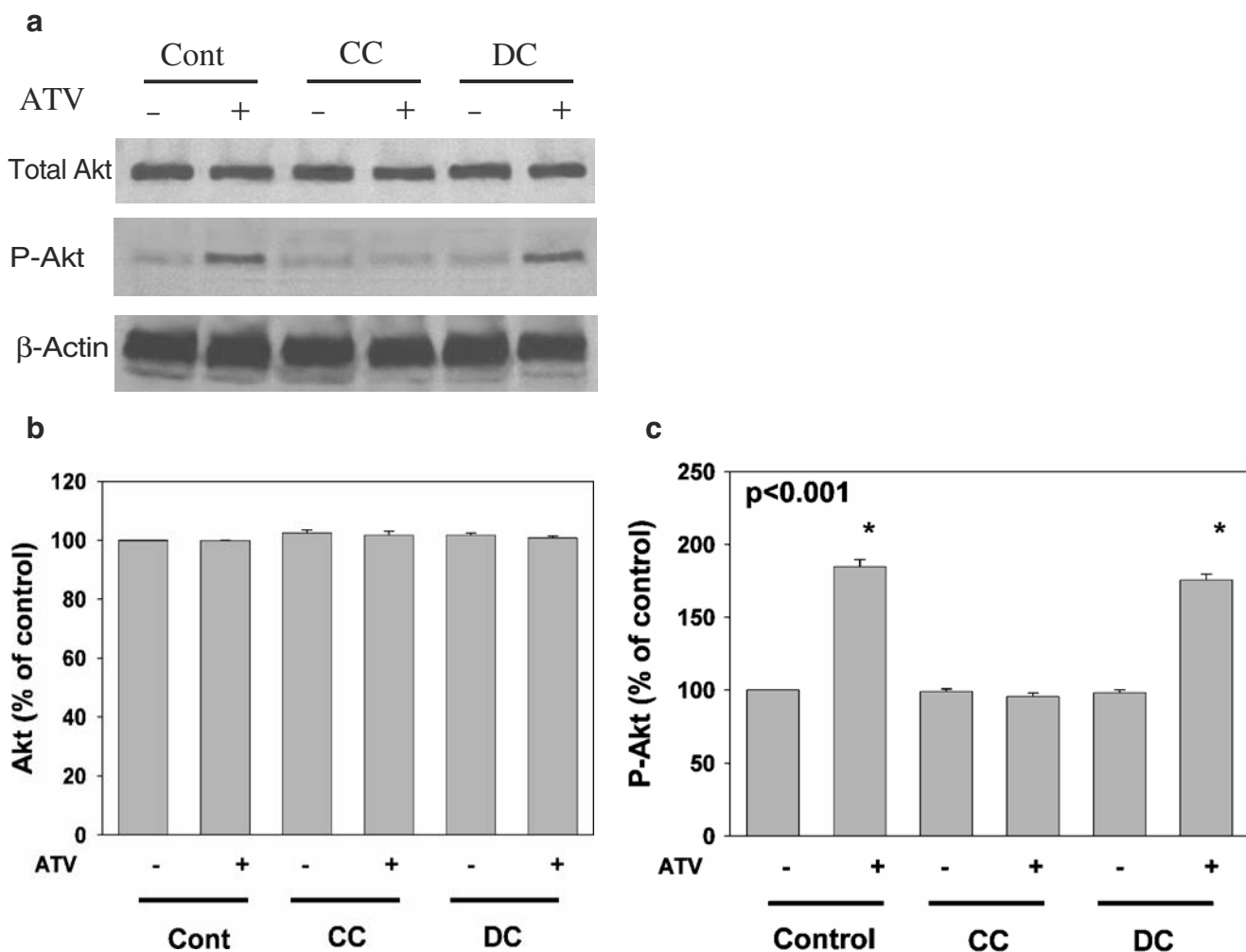

Fig. 4 Representative immunoblots (a) and densitometric analyses of myocardial levels of total *Akt* (b) and Ser-473 P-Akt (c). *$p<0.001$ ATV versus no ATV

absorbed from the gastrointestinal tract and is metabolized in the liver via the cytochrome P450 system. Its half-life is 2.5 to 4.5 h but can be as long as 12 h, although its metabolism is fairly consistent for any given individual [37, 40]. With average daily intake of 3.1 cups of coffee/day, the daily consumption of caffeine may reach 4 mg/kg, a dose that is sufficient to block the ARs [37]. The reported plasma caffeine levels in humans consuming 5–8 mg/kg caffeine per day are 3–10 μg/ml [37, 40, 41]. The reported peak caffeine level after drinking one cup of CC is up to 2 μg/ml [37]. Thus, the caffeine concentrations found in our rats (5.99±0.11 μg/ml for CC and 1.73±0.01 μg/ml for DC) are within the range reported in humans. At concentrations occurring in regular daily consumption, caffeine binds with high affinity to A_1, A_{2A} and A_{2B} ARs and with lower affinity to A_3 ARs [7]. It is commonly believed that CC may abolish the vasodilator effect of adenosine and dipyridamole [42–44]. According to the ACC/AHA/ASNC guidelines, caffeinated beverages should be avoided for 24 h before adenosine or dipyridamole stress testing [44]. However, a recent study has challenged this belief and concluded that one cup of coffee does not reduce the sensitivity of adenosine single-photon emission computed tomography imaging for detection of ischemia [34].

Our study suggests that oral consumption of CC blocked the augmentation of Akt phosphorylation by ATV and thus, blocked the myocardial protective effects of statins against ischemia–reperfusion injury. Akt activation, with subsequent eNOS phosphorylation, is essential for mediating the myocardial protective effects of statins against ischemia–reperfusion injury [14, 45–49]. We have recently shown that both theophylline and 8-SPT, non-specific AR inhibitors, blocked ATV induction of ERK 1/2, Akt and eNOS phosphorylation in the rat heart [25]. Moreover, both dipyridamole [32] and cilostazole [50] augment the infarct-size limiting effect of low-dose ATV by augmenting myocardial levels of adenosine. In addition to their protective effects against ischemia–reperfusion injury, Akt and eNOS activation have central roles in mediating other pleiotropic effects of statins, such as vasodilation, anti-inflammatory effects, anti-platelet effects, anti-atherosclerosis effects, mobilization of endothelial progenitor cells and neovascularization [51–53]. Thus, heavy consumption of CC may deprive patients from these potentially important non-lipid lowering effects of statins.

In our study CC had no deleterious effects when given to control rats without pre-existing atherosclerosis, although it prevented the protective effects of ATV. As caffeine blocks also the protective effects of ischemic preconditioning [5], CC may interfere also with the intrinsic protective mechanisms of the heart and thus, may be particularly deleterious in patients at high risk for ischemic events and in those receiving statins therapy for the prevention of

atherosclerosis. This is in agreement with the findings of Azevedo and Barros, who found that CC is associated with a risk of acute myocardial infarction only in patients with a family history of myocardial infarction [54]. On the other hand, in our study short term exposure to DC had no effect on Akt phosphorylation and the infarct-size limiting effects of ATV. The average plasma caffeine level in the DC group was 1.73±0.01 μg/ml, significantly lower than in the CC group. The fact that DC did not block Akt phosphorylation and myocardial protection by ATV suggests that a critical concentration is needed for blocking the ATV effects.

In conclusion, oral consumption of CC, but not DC blocks the induction of Akt phosphorylation by ATV and abrogates its myocardial infarct-size limiting effect. Further studies should be conducted to assess whether consumption of CC has deleterious effects in patients receiving statin therapy.

Limitations IS in the present study is smaller than that reported by other groups after similar duration of ischemia. This might be related to the technique used to analyze IS (correction for each slice weight), animal age and size and other technical aspects. In our protocols we are using ketamine and xylazine to induce anesthesia and isofluorane to maintain it. Moreover, we are adding buprenorphine before chest closure. Isofluorane [55, 56] and opioid agonists [57] have shown to protect against ischemia–reperfusion injury and reduce IS. Although we added all agents after the beginning of ischemia, recent study have suggested that morphine has a postconditioning effects too [58].

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