Effects of acute caffeine administration on NOS and Bax/Bcl2 expression in the myocardium of rat

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Abstract

Caffeine is the most frequently ingested neuroactive drug in the world and it is largely used to delay fatigue and improve physical activity. Caffeine can modulate NO synthesis in cells and may influence muscular function by modifying the cellular cycle life-death. There is little data concerning the relationship between caffeine in the heart, NOS expression and apoptosis and no data regarding the acute effect of high doses of caffeine in the *in vivo* myocardium. We therefore studied hemodynamic NOS and Bax/Bcl2 expression in the rat myocardium after a single caffeine administration. Thirty-two male rats were divided into six groups: the first was iv-injected with caffeine (16 mg/kg), the second with caffeine + L-NAME (30 mg/kg), the third with caffeine + L-arg (0.5 g/kg), the fourth with caffeine + L-NAME + L-arg and finally the fifth with saline. Mean arterial blood pressure (MAP) was monitored for 30 min, then the animals were killed. The sixth group was injected with caffeine and killed after 2 h. The hearts were isolated and processed by immunohistochemistry. We found that caffeine increased MAP temporarily while caffeine + L-NAME increased it for a longer period. In the control myocardium, all NOS isoforms were expressed. The Bcl2 were strongly expressed inside the perinuclear cytoplasm whereas Bax was very faintly detectable in the peripheral cytoplasm. In caffeine and caffeine + L-NAME treated animals, NOS expression disappeared. Bax and Bcl2 expression did not vary. The L-arg administration reversed these caffeine and L-NAME effects on NOS expression. Two hours after caffeine, NOS expression increased and Bax and Bcl2 expression did not vary, although Bcl2 was mainly expressed in the peripheral cytoplasm. We conclude that improved caffeine-induced physical performance could also be related to caffeine's ability to interfere with endogenous myocardial NO synthesis. Furthermore, we suggest that myocardial cell plays an effective anti-apoptotic role against acute caffeine administration.

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1. Introduction

Caffeine (1,3,7-trimethylxanthine) is one of the most common constituents of various beverages and is very probably the most frequently ingested neuroactive drug in the world [1]. Caffeine is mainly found in tea and coffee; a cup of coffee contains up to 75–150 mg caffeine (corresponding to about 1–2 mg/kg b.w.). In humans an oral dose of 1 mg/kg caffeine can yield plasma caffeine levels of 5–10 μmol/L [2]. A typical daily caffeine intake in humans is 3 mg/kg; so the daily consumption of several cups of coffee and/or tea could cause high plasma levels of caffeine that could be dangerous for certain physical conditions.

Caffeine is a lipophyl molecule that is capable of rapidly crossing the biological membrane. It is adsorbed at a gastrointestinal level reaching maximum blood concentration after 15 min to 2 h, and subsequently decreases until it completely disappears. Its persistence inside the organism is short and there is no accumulation. Caffeine is commonly used as a stimulant to prevent sleepiness and it is a component of several over-the-counter medications including certain pain remedies and in addition caffeine is widely used to improve physical activity. The energising effects of caffeine to improve physical and athletic performance are dose-dependent [3,4,5,6]. In humans, a single oral dose of caffeine (400 mg corresponding to about

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5 mg/kg b.w. or 5 coffees) maintain physical performance during an overnight period of sleep loss [4]. Previous studies on murine experimental models show that the injection of 16 mg/kg of caffeine increased physical activity, whereas overdoses decreased it [7].

The cardiac myocyte is the most energetic cell in the body contracting constantly throughout its lifespan, about 3.8 billion times, apparently without tiring or regenerating. Recent studies show that acute caffeine ingestion increases systolic blood pressure dose-dependently without affecting the resting heart rate [5]. Current evidence supports the hypothesis that caffeine influences the cardiovascular system at least acutely, not only by increasing peripheral blood pressure but also by increasing arterial stiffness and increasing arterial wave reflections. Moreover, it now seems that previous peripheral pressure measurements may have underestimated caffeine pressure effects, as a significantly greater response has been observed in aortic pressure [8].

Caffeine acts on various organs, in particular central nervous system [9], skeletal muscles [10] and cardiac muscle [5,11,12], and many hypotheses have been put forward to explain its mechanism.

Physiological studies have shown a significant decrease in exhaled NO after caffeine ingestion [13] demonstrating that caffeine can modulate NO synthesis in cells [7]. NO is a short-lived ubiquitous gas molecule generated from L-arginine degradation (L-arg) to citrulline, by the activation of enzyme NOS Ca^{2+}/calmodulin-dependent [14]. There are multiple NOS isoforms, e.g., neuronal-NOS (nNOS or type I), inducible-NOS (iNOS or type II), endothelial-NOS (eNOS or type III) [15].

NO modulates cardiac function which influences Ca^{2+} channels critical to excitation–contraction coupling [16]. Indeed, NO inhibits L-type Ca^{2+} channels in the heart but stimulates sarcoplasmic reticulum (SR) Ca^{2+} release, leading to variable effects on myocardial contractility. In particular nNOS and eNOS mediate independent, and in some cases opposite, effects on cardiac structure and function. Barouch et al. [17] showed that spatial confinement of specific NOS isoform regulates this process. eNOS is localised in the claveolae, where the compartmentalisation with β-adrenergic receptors and L-type Ca^{2+} channels allows the NO to inhibit β-adrenergic-induced inotropy, and so inhibits contractility. On the contrary, nNOS targets cardiac SR, and NO stimulation of SR Ca^{2+} release via the ryanodine receptor *in vitro*, suggests that nNOS may improve the contractility.

Many forms of inflammation are associated with increased NO production owing to the induction of iNOS gene, indeed iNOS expression increases greatly in skeletal muscle cells of patients with chronic heart failure, autoimmune inflammatory myopathies or inflammatory cytokines [18]. iNOS expression leads to continuous production of large amounts of NO and many studies have found that when cardiac myocytes express iNOS, the contractile function is decreased [19,36]. Therefore, iNOS inhibition could be an important target to increase myocardium contractility and reduce myocardial damage.

Over the last decade, increasing evidence has suggested that apoptosis is an important mechanism involved in the devel-opment and progression of cardiovascular disease. Apoptotic processes are regulated by several proteins including, Bax and Bcl2 which both play important roles. Indeed, Bcl2 and Bax expression is a critical intracellular checkpoint of apoptosis within a distal common cell death pathway. Over-expression of Bcl2 promotes cells survival *in vitro* and *in vivo* [20,21,22]. When Bax is over-expressed, apoptosis death is accelerated. Thus, the ratio Bcl2/Bax is important in determining the susceptibility to apoptosis and whether the cells survives or dies [23]. In the myocardium, apoptosis has been detected in a number of cardiac pathologies including hypoxia, ischemia followed by reperfusion, myocardial infarction, myocardial hypertrophy, and more recently, in patients with end-stage heart failure [24]. Jafari and Rabbani [25] showed that caffeine may stimulate the muscular apoptosis dose-dependently: low caffeine concentration (less than 50 μmol/L) prevents apoptosis, whereas higher concentration stimulates it. These data suggest that caffeine may influence muscular function modifying the cellular life-death cycle. However, there is little data concerning the relationship between caffeine and apoptotic mechanisms in the heart and until now no data regarding the acute effect of high doses of caffeine on apoptosis of myocardium *in vivo*. Therefore, in this study we tested the hypothesis that intravenous administration of a high single dose of caffeine plays a primary role in NOS and apoptotic protein expression (Bax/Bcl2) inside the rat myocardium. We performed the experiments with or without the non-selective and potent inhibitor of NOS (L-NAME) and/or an NO precursor (L-arg).

2. Materials and methods

The work was carried out according to National Animal Protection Guidelines. Thirty-two male Sprague–Dawley rats (mean body weight, 250 ± 20 g) were maintained with a standard diet and with water *ad libitum*. They were placed in a quiet room with controlled temperature and humidity. A 12/12 h light–dark cycle was maintained (7 a.m. to 7 p.m.). All experiments were performed at the same time and in the afternoon light period (2–5 p.m.). Caffeine, L-NAME and L-arg were purchased from Sigma (Milan, Italy) and were prepared immediately before the experiments by being dissolved in 0.9% saline.

2.1. Animal preparation

The animals were randomly assigned to each drug regimen and divided into six groups: (I) the saline-treated controls ($n = 5$); (II) caffeine treated ($n = 7$); (III) caffeine treated plus L-NAME ($n = 5$); (IV) caffeine treated plus L-arg ($n = 5$); (V) and caffeine treated plus L-NAME plus L-arg ($n = 5$) and (VI) intravenously injected into the caudal vein with caffeine ($n = 5$). The animals were anaesthetised with sodium pentobarbital (30 mg/kg, i.p.). Tracheotomy was performed, and the animals were mechanically ventilated (Ugo Basile, rodent ventilator 7025) to ensure physiological levels of arterial blood gases (P_{O_2}, 70–80 mmHg; P_{CO_2}, 27–35 mmHg). A single-lumen catheter was placed into the right carotid artery to record blood pressure, and a second catheter was placed into the right jugular vein for drug infu-

sion. According to the groups, about 1 ml of drugs solution were injected into the jugular or tail vein in each animals.

2.2. Experimental protocol

The detailed experimental protocol has been previously described [26]. Briefly, a single high dose of caffeine (16 mg/kg), was administered intravenously (volume injected 0.3 ml). This dose was chosen on the basis of previous experimental work on murine models [7].

Mean arterial blood pressure (MAP) was monitored and recorded throughout the experiments with a polygraph (O.T.E., C6b Recorder). NO-dependent vascular tone was assessed by infusing 30 mg/kg L-NAME (volume injected 0.3 ml), a competitive inhibitor of NOS [27], 3 min after caffeine infusion. After 5 min, 0.5 g/kg of L-arg (volume injected 0.4 ml) was administered to counteract L-NAME blockage. In addition, two groups were infused with caffeine and L-NAME or L-arg only.

The animals that received caffeine infusion only, after 3 min and 5 min were also injected with the same volume of saline used for L-NAME and L-arg infusion (about 0.3 ml and 0.4 ml, respectively). The animals that received caffeine and after 3 min L-NAME or L-arg infusion, after 5 min were also injected with saline (about 0.4 ml and 0.3 ml, respectively). Control rats received an intravenous bolus of saline of the same volume of the drugs. So the volume of injected solution and the modality of infusion were the same in all groups. After 30 min of hemodynamic records, animals belonging to the first five groups were killed. The sixth group was injected intravenously in the tail vein with caffeine and killed after 2 h under deep anaesthesia with no hemodynamic registration. The heart was quickly removed and placed in ice-cold saline solution, then frozen in liquid nitrogen and stored at $-80\,^{\circ}$C.

2.3. Immunohistochemistry

Cryo-sections of myocardium (7–10 μ thick) were processed by standard immunohistochemistry procedures as previously described [26]. Briefly, they were incubated overnight with polyclonal antibodies anti-nNOS, anti-iNOS o anti-eNOS (Chemicon International) and anti-Bcl2 or anti-Bax (Santa Cruz Biotechnology). Then, the sections were washed in PBS, incubated with byotinilated goat–anti-rabbit IgG (Dako, Milan, Italy), and incubated in avidin–biotin horseradish peroxidase complex (ABC-kit, from Dako) according to the manufacturer's instructions. The ABC complex was visualised by 1 mg/ml of diamminobenzidine (DAB) in PBS with 150 μl of 3% H_2O_2. The sections were counterstained with hematoxiline and mounted on glass slides.

Staining intensity was graded as, ($-$) when staining was absent, ($\pm$) when staining was barely visible, (+) when staining was faint, (++) when staining was moderately positive, (+++) when staining was clearly positive and strong. Two uninformed observers, analysed the sections from each animal of the different groups.

Negative control experiments were carried out by incubating the myocardium sections in PBS without the primary antibodies. The specificity of antibody labelling was investigated using appropriate controls, incubating the tissue sections with non-immunised goat serum for NOS (I–III), Bax and Bcl2 or PBS instead of the primary or secondary antibodies or ABC complex.

2.4. Statistics

The hemodynamic registration data were expressed as mean ± standard deviation (S.D.). Comparison of sample groups were made by ANOVA and Student–Newman–Keuls test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hemodynamics

The results are summarised in Table 1. In all treated groups, the heart rate (HR) decreased slightly. After infusions of caffeine, MAP increased immediately about 20%, but quickly

Table 1
Heart rate (HR) and mean aortic pressure (MAP) measurements in treatment protocol

	Control $n = 5$	Caffeine $n = 7$	Caffeine + L-NAME $n = 5$	Caffeine + L-Argn $= 5$	Caffeine + L-NAME + L-Arg $n = 5$
HR (pulse/min)	330 ± 11	310 ± 29	315 ± 24	310 ± 23	315 ± 35
S–N–K test		ns	ns	ns	ns
MAP (mmHg)	88.25 ± 7.7	105.71 ± 6.8	124.67 ± 4.5	56.67 ± 2.9	49.21 ± 3.2
S–N–K test					
1 vs 2	$q = 7.641$	$p < 0.05$	–	–	–
1 vs 3	$q = 14.756$	–	$p < 0.05$	–	–
1 vs 4	$q = 12.795$	–	–	$p < 0.05$	–
1 vs 5	$q = 15.817$	–	–	–	$p < 0.05$
2 vs 3	–	$q = 8.297$	$p < 0.05$	–	–
2 vs 4	–	$q = 21.461$	–	$p < 0.05$	–
2 vs 5	–	$q = 24.725$	–	–	$p < 0.05$
3 vs 4	–	–	$q = 27.550$	$p < 0.05$	–
3 vs 5	–	–	$q = 30.573$	–	$p < 0.05$
4 vs 5	–	–	–	$q = 3.022$	$p < 0.05$

S–N–K = Student–Newman–Keuls test; ns = not significant.

Table 2
Intensity of immuno-reactivity anti-NOS in myocardial fibres in differently treated groups

	nNOS	iNOS	eNOS
Control (saline)	++	+++	+++
Caffeine 30′	−	−	+
Caffeine 2 h	+++	+++	+++
Caffeine + L-NAME 30′	−	−	+
Caffeine + L-arg 30′	++	++	++
Caffeine + L-NAME + L-arg	++	++	++

Table 3
Intensity of immuno-reactivity anti-Bax/Bcl2 in myocardial fibres in differently treated groups

	Bax	Bcl2
Control (saline)	−/+	+++
Caffeine 30′	+	+++
Caffeine 2 h	+	+++
Caffeine + L-NAME 30′	−/+	++
Caffeine + L-arg 30′	−/+	+++
Caffeine + L-NAME + L-arg	−/+	+++

returned to the base level. The administration of L-NAME after caffeine increased MAP by about 18% during the whole recording. The administration of L-arg after caffeine decreased MAP about 36%, but after 30 min MAP reached its base level. Administration of L-NAME and L-arg after caffeine, decreased MAP about 44%, and after 30 min MAP was near its normal level.

3.2. Immunohistochemistry

3.2.1. Controls
3.2.1.1. Nitric oxide synthase. The results are summarised in Table 2. Inside the myofibres all NOS isoforms were expressed. However, a different intensity of NOS isoform distribution was observed; nNOS immuno-staining was moderately expressed mainly in the periphery of the fibres (Fig. 1A), whereas iNOS and eNOS were strongly expressed inside central perinuclear cytoplasm only (Fig. 1D and 1G).

3.2.1.2. Bax and Bcl2. The results are summarised in Table 3. Bcl2 was strongly expressed inside the central cytoplasm of the fibres (Fig. 2A). The Bax expression was very faintly detectable in the fibres' periphery (Fig. 2D).

3.2.2. Caffeine
3.2.2.1. Nitric oxide synthase. The results are summarised in Table 2. After 30 min from caffeine infusion, the nNOS and iNOS expression disappeared or was very faintly expressed (Fig. 1B and E), whereas the eNOS was faintly expressed (Fig. 1H). Two hours after caffeine administration, all NOS immuno-reactivity increased considerably mainly in the periphery of the fibres (Fig. 1C, F, I). L-NAME administration after caffeine infusion deleted nNOS and iNOS expressions whereas eNOS was faintly expressed. L-arg administration after caffeine infusion, caused a moderate NOS expression inside the myofibres like the control ones. The administration of L-NAME and L-arg after caffeine, restored control parameters.
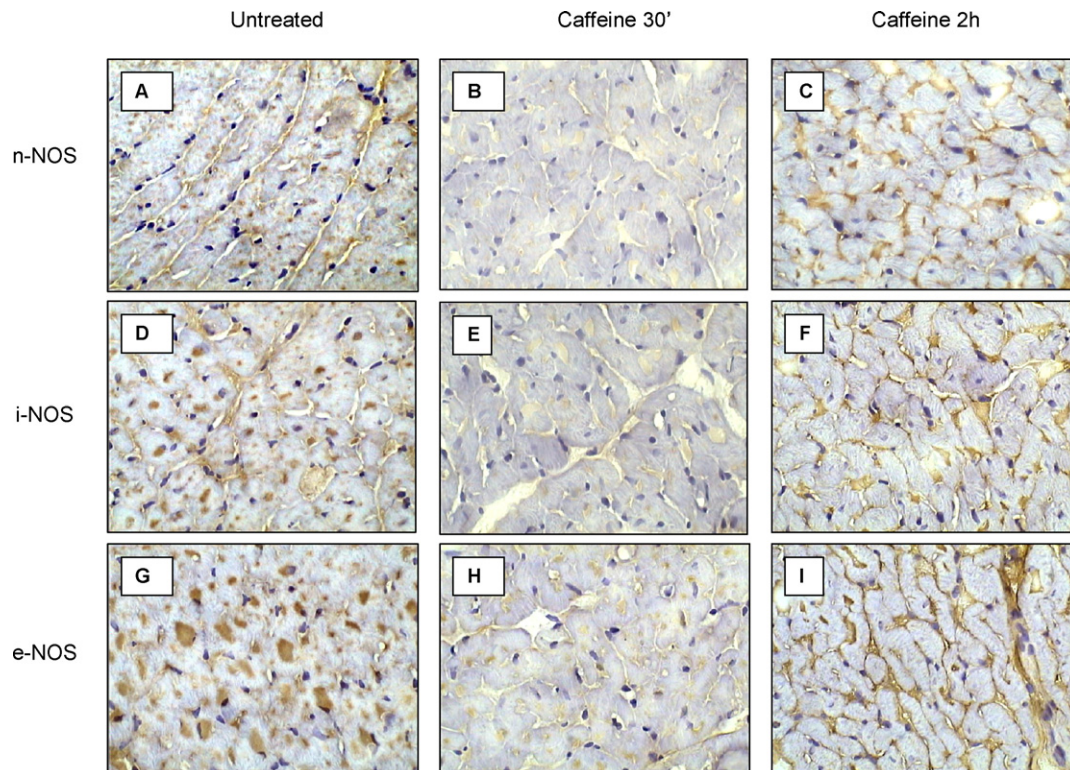

Fig. 1. NOS expression in untreated (A, D, G) and caffeine treated animals after 30′ (B, E, H), and 2 h (C, F, I). Magnification 20×.
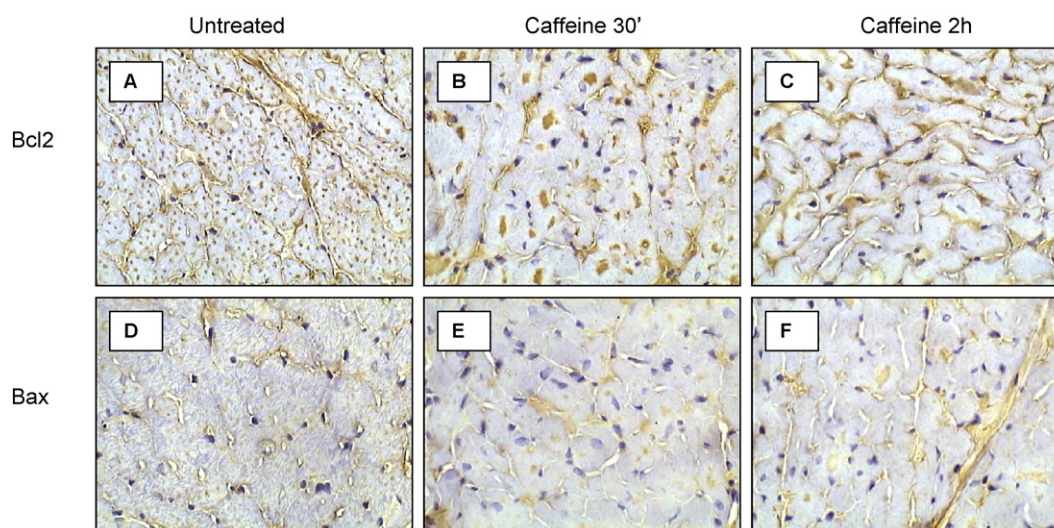
Fig. 2. Bcl2 and Bax expression in untreated (A, D) and caffeine treated animals after 30′ (B, E), and 2 h (C, F). Magnification 10× (A, D) and 20× (B, C, E, F).

3.2.2.2. Bax and Bcl2. The results are summarised in Table 3. After 30 min from caffeine infusion, Bax immuno-staining of the myocardial cell was faint (Fig. 2E). Bcl2 was strongly expressed inside central cytoplasm in the same way as controls (Fig. 2B). After 2 h from caffeine administration Bax and Bcl2 expression did not vary (Fig. 2F and C, respectively). However, the Bcl2 immuno-reactivity was mainly expressed in the periphery of the fibres (Fig. 2C).

L-NAME administration after caffeine infusion caused a strong Bcl2 expression like that observed after caffeine administration only and distributed in the periphery of the fibres. Bax was very faintly expressed.

L-arg administration after caffeine infusion did not vary the Bcl2 expression with distribution similar to untreat animals. The Bax expression was absent or very scarce.

The administration of L-NAME and L-arg after caffeine, restored control parameters.

4. Discussion

The main findings of our study after a single high dose of caffeine were: (1) caffeine increased MAP quickly but for a short time without influencing HR; (2) caffeine inhibited the expression of all NOS isoforms inside the myocardium. This effect was transitory and disappeared 2 h after treatment; (3) caffeine did not vary the Bax/Bcl2 expression and so it did not induce apoptosis.

4.1. Hemodynamic

Single high dose of caffeine induced only a slight HR decrease in all animal groups and this was probably due to anaesthesia effects according to Pasini et al. [28].

A previous human study showed that caffeine ingestion (5 or 10 mg/kg body weight) increased resting systolic pressure in a dose-dependent fashion but these effects on blood pressure were not maintained during 30 min of moderately intense cycling exercise (60% VO_2 peak) [5]. Rats with chronic oral caffeine administration (0.2% in drinking water) for 2 weeks increased the resting heart rate and blood pressure, which were concurrent with changes in adenosine receptor function [29]. The hemodynamic data emerging from our study showed the quickly increase of MAP, agreed with these reports. In addition they showed that a single high dose of caffeine promptly increases the MAP, but the effect is very short and so NO independent. Indeed, treatment with inhibitor of NOS L-NAME increases the MAP duration whereas injection of L-arg counteracts L-NAME effects. So we suggest that the transient caffeine hemodynamic effects probably do not influence cardiac performance.

4.2. Nitric oxide synthase

NO is an important regulator of muscle contractility and attenuates cardiac myocyte contraction [30]. Recently, Xu et al. [30] suggested that a cardiac muscle nNOS isoform is located on SR of cardiac myocytes, where it may respond to intracellular Ca^{2+} concentration and modulates SR Ca^{2+} ion active transport in the heart.

nNOS is targeted to cardiac SR [17]. Khan et al. [31] demonstrated that nNOS selectively regulated the cardiac force–frequency response via influence over SR Ca^{2+} cycling. Trafford et al. [32], in isolated rat ventricular myocytes, showed that low concentrations of caffeine increased the frequency of spontaneous release of Ca^{2+} by SR. However, caffeine had only transient effects on stimulated Ca^{2+} release produced by depolarising pulses, so its effect was short. In this study, we showed that acute caffeine administration briefly inhibited nNOS expression so only temporarily influenced the cardiac force–frequency.

A previous study showed that eNOS immuno-staining was present in endothelial cells of capillaries of the conducting and working myocardium and endocardial cells [33]. Our data confirmed that eNOS constitutionally expressed inside the myocardium was transitorily inhibited by caffeine administration. eNOS is a dually acylated peripheral membrane protein that

targets the Golgi region and claveolae of endothelial cells [34]. Petroff et al. [35] implicated eNOS in stretch induced cardiomyocyte Ca^{2+} transients. More recently, Barouch et al. [17] showed that eNOS was localised in the claveolae, where compartmentalisation with β-adrenergic receptors and L-type-Ca^{2+} channels allowed NO to inhibit β-adrenergic-induced inotropy reducing contractility. Indeed, these authors demonstrated that eNOS deficient mice had increased contractility. So we can speculate that inhibition of eNOS expression caffeine-induced observed in our experiments may transitorily increase the contractility of myocardium.

Many studies have shown that iNOS expression and NO production decreases contractile function causing myocardial dysfunction [36]. Ziolo et al. [37], showed that NO produced via NO donors and endotoxin-induced iNOS expression, depresses the β-adrenergic response in human myocardium. This mechanism could be an important signalling pathway in cardiomyopathies, including human heart failure. In fact, there is a significant correlation between heart failure severity and NO production. Therefore, the inhibition of iNOS expression after caffeine administration that we observed may be a beneficial for myocardial cells.

The inhibition of iNOS after acute caffeine treatment is short, and after 2 h iNOS expression increases more than base expression suggesting probable myocardial damage. However, recently, Jones and Bolli [38] proposed that iNOS expressed in cardiac myocytes is a profoundly protective protein. They suggested that NO iNOS-derived interacts with components of the electron transport chain and/or the mitochondria permeability transition pore to limit post-ischemic myocardial damage and this action potentially provides a fundamental molecular explanation for the mechanism of NO-mediated cardioprotection. At present, we do not know the precise role of NO iNOS-derived on myocardial function after single caffeine administration and further studies in chronically caffeine treated animals are necessary to understand the NO iNOS-derived effects and/or its intracellular target.

4.3. Bcl2 and Bax

The Bcl2 family protein is either proapoptotic (Bax, Bid, Bik, Bak) or anti-apoptotic (Bcl2, BclX). Activation of the proapoptotic members causes their translocation to the mitochondria where they interact with anti-apoptotic members that are components of the mitochondria membrane [39]. This interaction depolarises voltage-dependent mitochondria channels and releases mitochondria mediators of apoptosis such as cytocrome c [40].

In *in vitro* studies, caffeine has been reported to affect cell cycle function, inducing programmed cell death or apoptosis and perturbing key cell cycle regulatory proteins. Caffeine concentration <1 mM induced p53-dependent apoptosis associated with increased expression of pro-apoptotic Bax and caspase-3; at concentrations of 1–2 mM caffeine could induce G1 arrest, whereas concentrations 2–4 mM induced apoptosis and blocked G1 arrest [41]. Physiologically, the simultaneous consumption of over 100 cups of coffee is needed for a 2 mM blood level of caffeine [42]. This dosage is similar to that used in our experiments, however we did not observe any signs of apoptosis. This difference could be due to our different technical approach, especially as our experimental model was *in vivo*.

Apoptosis has been detected in the myocardial fibres in a number of cardiac pathologies including hypoxia, ischemia followed by reperfusion, myocardial infarction, hypertensive cardiomyopathy, myocardial hypertrophy, and in patients with end-stage heart failure [24]. However, there are very little data regarding the apoptosis after acute caffeine consumption *in vivo*. In our previous work performed on skeletal muscle, we showed that acute caffeine administration caused a transitory decrease of anti-apoptotic Bcl2 expression [26]. Curiously, we observed that i, Bcl2 expression in the heart after caffeine infusion did not vary. This suggests an intrinsic protective mechanism in the myocardial fibres. We hypothesise that the difference between skeletal muscle and myocardial fibres could be explained by the fact that the latter reduced their regenerative properties. Moreover, only a few undifferentiated stem cells are present in the myocardium [43]. Vlachopoulos et al. [44] showed in humans who were administered about 3 mg/kg caffeine acute heart toxicity and death did not occur. The same results were obtained in orally rats administered with 30 mg/kg caffeine [45]. However, this caffeine dosage was only about 1.4× than of an average human exposure (3 mg/kg) based on a mg/m^2 body surface-area comparison [46]. Therefore, our data showed that even a single very high dose of caffeine does not seem to have any pro-apoptotic properties on cardiomyocytes *in vivo*.

5. Conclusions

Our data demonstrated that a single high dose of caffeine rapidly reduces NOS expression in myocardial fibres. This effect could be related to the ergogenic properties of caffeine on the myocardium. In addition, unlike the skeletal muscle, a high dose of caffeine does not vary the apoptotic protein expression inside myocardial cells which would suggest an effective protective mechanism inside the myocytes. Further studies will be carried our to monitor the effects of chronic caffeine treatment on NOS and Bcl2 expression and so to find the role of caffeine in influencing cardiac performance.

Acknowledgements

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