High plasma concentrations of asymmetric dimethylarginine inhibit ischemic cardioprotection in hypercholesterolemic rats

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Abstract

A low concentration of nitric oxide associated with a high concentration of asymmetric dimethylarginine (ADMA) can explain the lack of ischemic cardioprotection observed in the presence of hypercholesterolemia. The objective of the present study was to evaluate the effect of hypercholesterolemia on ischemic pre- and postconditioning and its correlation with plasma concentrations of ADMA. Male Wistar rats (6-8 weeks old) fed a 2% cholesterol diet (n = 21) for 8 weeks were compared to controls (n = 25) and were subjected to experimental myocardial infarction and reperfusion, with ischemic pre- and postconditioning. Total cholesterol and ADMA were measured in plasma before the experimental infarct and the infarct area was quantified. Weight, total cholesterol and plasma ADMA (means ± SE; 1.20 ± 0.06, 1.27 ± 0.08 and 1.20 ± 0.08 *vs* 0.97 ± 0.04, 0.93 ± 0.05 and 0.97 ± 0.04 µM) were higher in animals on the hypercholesterolemic diet than in controls, respectively. Cardioprotection did not reduce infarct size in the hypercholesterolemic animals (pre: 13.55% and post: 8% compared to 7.95% observed in the group subjected only to ischemia and reperfusion), whereas infarct size was reduced in the animals on a normocholesterolemic diet (pre: 8.25% and post: 6.10% compared to 12.31%). Hypercholesterolemia elevated ADMA and eliminated the cardioprotective effects of ischemic pre- and postconditioning in rats.

Key words: Hypercholesterolemia; Cardiotonics; Nitric oxide; Risk factors; Asymmetric dimethylarginine

Introduction

The literature is not unanimous about the influence of hypercholesterolemia on the infarct size after ischemic pre- and postconditioning. In hyperlipidemic patients subjected to coronary angioplasty, the loss of the beneficial effect caused by classical ischemic preconditioning is correlated with increased levels of total cholesterol (TC) and cholesterol associated with low-density lipoprotein (LDL) cholesterol (1). Additionally, the limiting effect of infarct size caused by this form of cardioprotective ischemia is attenuated in rabbits fed a diet enriched with up to 1% cholesterol (2). However, there are reports based on research with animals that did not demonstrate an influence of hyperlipidemia on the beneficial effects of classical ischemic preconditioning (3-6).

The effects of hyperlipidemia on myocardial postconditioning have been studied less, possibly due to the fact that this form of protection has only been studied recently. Some studies have shown the lack of beneficial effects of postconditioning in animals with experimental hyperlipidemia (3). In contrast, Donato et al. (7) showed a reduction of myocardial damage in a model of experimental myocardial infarction and hypercholesterolemia involving rabbits subjected to ischemic postconditioning.

The role of nitric oxide (NO) and asymmetric dimethylarginine (ADMA)

A reduction in the bioavailability of NO due to increased nitrosactive stress, among other mechanisms, can contribute to the loss of the effect of myocardial preconditioning in some hyperlipidemic animals (2). In classical preconditioning, endogenous NO does not appear to play an important role, whereas exogenous NO causes myocardial protection (8). In late preconditioning, which occurs 24 to 96 h after sublethal ischemia, experimental studies have indicated that hypercholesterolemia suppressed the protection of the myocardium

induced by NO (9). NO generated by endothelial NO synthase (eNOS) triggers a cascade of molecular events that culminate in the late activation of inducible NOS that provides cardioprotection (10).

A study that applied postconditioning showed a lack of protection when NOS was inhibited by L-NAME (11). This result shows that NO, probably generated by eNOS, is necessary for the limitation of myocardial necrosis, with its relevant clinical effects, provoked by postconditioning.

The inhibition of NOS and consequent endothelial dysfunction is obtained in animals with induced atherosclerosis (12,13). In humans, evidence shows a positive correlation between the blood concentrations of ADMA and cholesterol (14). However, some studies did not find this correlation (15).

Vegh et al. (16) reported that the beneficial effect of ischemic preconditioning to avoid arrhythmias induced by ischemia and reperfusion during the first window of myocardial protection was eliminated by L-NAME. Penna et al. (17) demonstrated that L-NAME infusion for 3 min completely evaded the protection induced by postconditioning. The systemic administration of NOS inhibitors during ischemia and just before reperfusion caused an increase in infarct size, an effect that could be reversed by the administration of L-arginine (18).

The objective of the present study was to evaluate the effect of hypercholesterolemia on the infarct area in rats subjected to ischemia and reperfusion, alone or associated with ischemic pre- and postconditioning, and high plasma concentrations of ADMA.

Material and Methods

Male Wistar rats (*Rattus norvegicus*) aged 6 to 8 weeks were used. The animals were kept at room temperature, varying from 22° to 24°C, on a 12-h light-dark cycle.

Control animals (n = 25) were fed a normocholesterolemic diet (Nuvilab Nutrientes S/A, Brazil) while experimental animals (n = 21) were fed exclusively a diet enriched with 2% cholesterol, provided by Nutri Experimental®, Brazil, for a period of 8 weeks (Table 1). In previous studies (19), similar diets also increased the levels of cholesterol.

All animals were subjected to an experimental myocardial infarct (20) by means of occlusion of the anterior descending coronary artery (ADA), for a period of 30 min, followed by reperfusion (open artery).

Three groups of animals were used as controls. The ischemia/reperfusion group (I/R) was subjected just to ADA occlusion for 30 min (lethal ischemia), followed by reperfusion. The I/R with preconditioning group (PC) (21) was subjected to three periods of 3 min of ischemia, interspersed with an equal period of reperfusion, before lethal ischemia. The I/R with postconditioning group (POC) (22) was subjected to 3 periods of 10 s of ischemia

Table 1. Composition of the hyperlipidic diet used in the intervention group.

Component	Quantity (%)
Carbohydrates*	51.95
Protein source#	20.00
Lipids+	18.00
Fibers	5.00
Mineral mix	1.00
Vitamin mix	3.00
L-cysteine	0.30
Choline bitartrate	0.25
Cholic acid	0.50
Total	100.00

*Carbohydrate fractions: 55.8% starch, 25.8% malt dextrin, and 18.3% sucrose. #Protein source = commercial casein (85% protein). +Lipid fraction = 66.7% coconut oil, 26.4% soybean oil, and 6.9% cholesterol. The vitamin and mineral mixtures followed the recommendations of the American Institute of Nutrition (AIN-93G).

interspersed with equal periods of reperfusion in the first minute after lethal ischemia. The other 3 groups that received a cholesterol-rich diet were subjected to the same protocols (I/R-HYPER, PC-HYPER and POC-HYPER).

Blood samples were collected before the experimental infarct by cannulating the right jugular vein and the blood concentrations of TC were analyzed using the trinder-enzymatic method (Labtest® Diagnóstico S/A, Brazil) and ADMA by ELISA (LDL-Diagnostika®, Germany).

The anesthetics xylazine (10 mg/kg) and ketamine (90 mg/kg) administered intraperitoneally, and 19.1% potassium chloride and 4% formaldehyde were used in this study, as described below.

After anesthesia, the animals were intubated with a 14-gauge polyethylene cannula and connected to a Harvard® mechanical ventilator, model 683 (Harvard Apparatus Inc., USA), with a current volume set at 1.1 mL/100 g body weight, and a respiratory frequency of 70-75/min. A polyethylene catheter (PE 50) was used to collect blood samples.

The fourth or fifth intercostal space was opened and the heart exposed. The ADA was occluded with a 6.0 polypropylene thread (Prolene®, Ethicon Inc., USA) at its proximal third, about 1 mm from the origin, pressing against a polyethylene catheter. After liberation, the thread was removed from the surgical field.

After the end of the procedure, the chest wall was closed (thread 4.0 Mononylon®, Ethicon Inc., Brazil) and the animals were placed in boxes heated with infrared lights and provided with supplemental oxygen for 1 to 2 h.

Eight days after acute myocardial infarction, with the animals anesthetized, an incision was made extending from the abdomen to the chest. The heart was exposed and a saline solution containing 14 mM KCl was

Table 2. Weight of the animals prior to experimental infarction.

Group	Mean ± SE (g)	SD	Minimum	Maximum
I/R (n = 10)	247.80 ± 13.81	43.64	171	307
PC (n = 7)	304.00 ± 10.93	28.86	273	347
POC (n = 8)	256.00 ± 22.70	64.03	170	366
I/R-HYPER (n = 8)*	409.00 ± 12.41	35.02	345	473
PC-HYPER (n = 5)*	451.20 ± 23.70	52.87	406	528
POC-HYPER (n = 8)*	391.38 ± 12.80	36.12	314	426

I/R = isolated ischemia/reperfusion; PC = I/R with preconditioning; POC = I/R with postconditioning; I/R-HYPER = I/R and hypercholesterolemia; PC-HYPER = I/R with preconditioning and hypercholesterolemia; POC-HYPER = I/R with postconditioning and hypercholesterolemia; n = number of animals. *$P < 0.05$ compared to the normocholesterolemic diet group (ANOVA).

administered under constant pressure of 80 mmHg, followed by 4% formaldehyde. The heart was excised and kept in formalin for 24 h. The hearts were then sectioned along the midpoint between basis and apex and a slice was embedded in paraplast for histological sections. Sections of 3 μM were stained with hematoxylin-eosin for qualitative assessment and with Masson's trichrome (blue stain) in the scar area, which was then measured by computerized planimetry (Leica Imaging Systems®, UK); the images were analyzed with a specific software (ImageQuant®, Leica®).

The variables were measured at a single time and are reported as means ± SE, SD and the minimum and maximum values. Group means were evaluated by ANOVA with classification factor. When significant results were obtained, the Tukey test was used to determine differences. The SPSS® software (SPSS Inc., USA) was applied and the level of significance was set at $P < 0.05$.

Results

Significant differences in the weight (Table 2) and plasma concentrations of cholesterol (Table 3) of the animals on the hyperlipidemic diet were observed compared with those on the normocholesterolemic diet.

Hypercholesterolemia contributed to increase the concentrations of ADMA (Table 4). A statistically significant difference was observed between the hypercholesterolemic and normocholesterolemic groups, whereas

no difference was observed between animals in the groups subjected to the same diet.

In the groups without hypercholesterolemia, pre- and postconditioning protected the myocardium with smaller infarcts; a fact not observed in the hypercholesterolemic animals (Table 5).

Discussion

A hyperlipidemic diet given to rats over a period of 8 weeks caused weight gain and increased concentrations of TC (Tables 2 and 3). However, the cholesterol concentrations detected were not as high as the levels observed by Matos et al. (19) even though the average weight gain was significant (Table 2).

The size of the infarct area considered was a percentage of the total transverse area of the left ventricle and not a percentage of the risk area. Triphenyltetrazolium was used to quantify the viable myocardium, as described by Dow and Kloner (23). Our infarction model with reperfusion and survival may also have contributed in some way to a lesser extent of myocardial necrosis, although reperfusion *per se* contributes to myocyte death (24).

Hypercholesterolemia did not influence the size of the infarcted area in the groups submitted to ischemia and reperfusion without myocardial protection. A smaller area of necrosis was observed, but without statistical significance (Table 5). This result is comparable to that obtained by Girod et al. (25) who studied rats fed a

Table 3. Total cholesterol of the animals prior to experimental infarction.

Group	Mean ± SE (mg%)	SD	Minimum	Maximum
I/R (n = 10)	69.50 ± 4.70	14.86	49	85
PC (n = 7)	73.71 ± 4.17	11.03	61	91
POC (n = 8)	75.25 ± 4.18	11.80	53	88
I/R-HYPER (n = 8)*	133.38 ± 8.65	24.40	109	181
PC-HYPER (n = 5)*	112.00 ± 5.88	13.13	94	123
POC-HYPER (n = 8)*	129.00 ± 10.72	30.24	104	198

For abbreviations, see legend to Table 2. *$P < 0.05$ compared to the normocholesterolemic diet group (ANOVA).

Table 4. Plasma ADMA of the animals prior to experimental infarction.

Group	Mean ± SE (µM)	SD	Minimum	Maximum
I/R (n = 10)	0.97 ± 0.04	0.13	0.81	1.18
PC (n = 7)	0.93 ± 0.05	0.14	0.77	0.19
POC (n = 8)	0.97 ± 0.04	0.14	0.76	1.18
I/R-HYPER (n = 8)*	1.20 ± 0.06	0.19	0.83	1.45
PC-HYPER (n = 5)*	1.27 ± 0.08	0.19	0.99	1.51
POC-HYPER (n = 8)*	1.20 ± 0.08	0.25	0.89	1.67

For abbreviations, see legend to Table 2. *$P < 0.05$ compared to the normocholesterolemic diet group (ANOVA).

cholesterol- enriched diet. Paradoxically, some investigators have shown a protective effect of hypercholesterolemia against myocardial damage secondary to ischemia and reperfusion (26,27).

When the cardioprotective effects were evaluated in the groups receiving a normocholesterolemic diet (Table 5), there was a smaller area of necrosis in the group subjected to both procedures (pre- and postconditioning). This result is comparable to those reported in the literature, which showed the benefits of these procedures for many species, including humans and rodents (23).

Beneficial cardioprotective effects were not observed in the hypercholesterolemic animals. The extension of myocardial damage was greater in the groups subjected to preconditioning (PC-HYPER) than in the groups without cardioprotection (I/R-HYPER), although without statistical significance (Table 5). The lack of ischemic cardioprotection is also observed in humans and correlates with the increased levels of TC and LDL cholesterol (1).

In contrast to our results, some literature reports have demonstrated that hyperlipidemia did not eliminate the favorable effects of classical myocardial preconditioning in the animals (3-6). The literature also shows that myocardial postconditioning acts beneficially even in hypercholesterolemic animals (7). However, some investigators have obtained results similar to ours, in that the tissue damage was not lower in hypercholesterolemic animals subjected to this form of modified reperfusion, which is cardioprotective (3).

The reduced bioavailability of NO can explain the lack of cardioprotection in the animals receiving a hyperlipidemic diet (2), as extensively reported regarding late myocardial preconditioning (10). However, the importance of NO in the classic form remains a subject for discussion (12). Regarding myocardial postconditioning, we also found evidence of a role of NO in the intrinsic cardioprotective mechanism (28).

The increased concentration of ADMA in the presence of hypercholesterolemia can be responsible for the low bioavailability of NO. The plasma concentrations of ADMA did not differ significantly between the normocholesterolemic groups, whose values, as expected, were within normal limits for the method used (29) (Table 4).

ADMA concentrations were significantly increased in the animals receiving the hypercholesterolemic diet compared to the animals receiving the normocholesterolemic diet, showing that the increased cholesterol levels contributed to the elevation of ADMA.

In our opinion, the elevation of ADMA and, indirectly, the blocking of eNOS represent a very reasonable hypothesis to explain the lack of beneficial effects of both pre- and postconditioning.

More studies are necessary, perhaps using a more appropriate animal model of endothelial dysfunction associated with pathological levels of this NOS inhibitor. An alternative would be to show a direct action of ADMA by injecting it into the animal at the time of ischemia and reperfusion, with and without cardioprotective measures.

Because the oxidized LDL is involved in ADMA elevation and is associated with reduced NO production and impaired endothelium-dependent vasodilation (30),

Table 5. Infarct size 8 days after experimental ischemia.

Group	Mean ± SE (%)	SD	Minimum	Maximum
I/R (n = 10)	12.31 ± 1.37	4.34	4.77	16.94
PC (n = 7)*	8.25 ± 1.87	4.95	2.40	18.15
POC (n = 8)*	6.10 ± 0.95	2.70	2.36	10.43
I/R-HYPER (n = 8)	7.95 ± 0.84	2.38	4.35	10.76
PC-HYPER (n = 5)	13.55 ± 1.16	2.60	10.54	16.64
POC-HYPER (n = 8)	8.00 ± 1.62	4.57	2.94	15.18

For abbreviations, see legend to Table 2. *$P < 0.05$ compared to the I/R group (ANOVA).

another alternative experimental model could be to inject the animals with lipoprotein modified with oxidants in order to cause an elevation of ADMA and the consequent NOS inhibition and endothelial dysfunction.

In conclusion, a hypercholesterolemic diet eliminated the cardioprotective effects of ischemic myocardial pre- and postconditioning in rats. In addition, according to our results, ADMA increased significantly and probably was involved in the lack of protection by both cardioprotective phenomena observed in this study.

Acknowledgments

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