Introduction
Alteration of myocardial endothelial nitric oxide synthase (eNOS) is classically involved in myocardial ischemia-reperfusion (IR) injury (Otani 2009), and then constitutes a privileged target for cardioprotection. Indeed numerous studies demonstrated attenuation of heart sensitivity to IR when eNOS pathway was improved by strategies such as eNOS overexpression (Brunner, Maier et al. 2003) (Szolled, Pokreisz et al. 2010) (Jones, Greer et al. 2004), NO administration or BH4 supplementation (Dumitrescu, Biondi et al. 2007). Among cardioprotective strategies, chronic exercise training is widely recognized (Powers, Quindry et al. 2008). Indeed, even if mechanisms of exercise induced preconditioning remained still partially unknown; increase of myocardial enzymatic antioxidant status is usually reported as the main factor of cardioprotection (Hamilton, Staib et al. 2003; French, Hamilton et al. 2008). Recently Calvert et al. (Calvert, Condit et al. 2011) demonstrated that eNOS-NO pathway is mainly involved in exercise-induced cardioprotection. In this study, authors reported that exercise protects the heart against myocardial IR by stimulation of β3-Adrenergic Receptors and increased cardiac storage of nitric oxide metabolites (nitrite and nitrosothiols). However, in this work, despite the role of eNOS is clearly pointed out, mechanisms responsible of eNOS-induced cardioprotection during post-ischemic reperfusion were not investigating.

The role of eNOS in myocardial IR is still misunderstood and controversial. Indeed in physiological state, this homodimeric enzyme leads to the formation of nitric oxide (NO) and L-citrulline, from oxygen and L-arginine, by electron transfer from monomer reductase domain to the other monomer oxygenase domain. The “coupled” state of eNOS, and thus its functional activity, is dependent of its cofactor tetrahydrobiopterin (BH4). Indeed, in lack of BH4, “uncoupled” eNOS generates superoxide (O2·−) instead of NO (Massion and Balligand 2003; Balligand, Feron et al. 2009). In myocardial post-ischemic reperfusion, the massive oxidative stress generated during early reperfusion is characterized by a burst of O2·− which can reacts itself with NO to form the extremely cytotoxic peroxynitrite (ONOO−) (Wang and Zweier 1996; Yasmin, Strynadka et al. 1997; Pacher, Beckman et al. 2007). ONOO− rapidly oxidizes BH4 to dihydrobiopterin (BH2), leading to the formation of the uncoupled eNOS (Chen, Druhan et al. 2010). Then, during post-ischemic reperfusion, eNOS uncoupling would play a major role in reperfusion injury through aggravation of oxidative stress and limitation of NO biodisponibility. Moreover, eNOS activity is also regulated post-translationally by phosphorylation of numerous enzymatic specific sites (Fleming 2010). Notably, phosphorylation of Ser1177 leads to an enhanced NO synthesis by the coupled enzyme, but has been reported as promoting O2·− formation when eNOS is uncoupled (Xia, Tsai et al. 1998; Chen, Druhan et al. 2008).

In addition, Chen et al. (2011) recently discussed about the possible necessity to produce O2·− rather than NO in pro-oxidant conditions in order to limit ONOO− formation. Indeed, whereas O2·− has a very short half life and can be rapidly scavenged in cardiomyocytes, ONOO− is recognized as a very persistent cytotoxic molecule (Ferdinandy and Schulz 2003; Pacher, Beckman et al. 2007).

In the present work we investigated then whether i) exercise training influenced eNOS function during cardiac IR, and ii) whether such alteration of eNOS phosphorylation/dimerization contribute to protect the myocardium against IR injuries. Surprisingly, we revealed that cardioprotection induced by exercise training against myocardial ischemia reperfusion injury requires eNOS uncoupling.

Materials and methods
Wistar male rats were randomly assigned to sedentary (Sed) or exercise group (Ex). Ex rats were submitted to 5 weeks of running, 5 days/week for 45 min/boots of exercise at ≈
70% VMA. Then a first set of animals were sacrificed to evaluate effect of exercise training on eNOS expression, phosphorylation, dimerization (low temperature electrophoresis), GTP-cyclohydrolase 1 (GCH1), and copper-zinc superoxide dismutase (CuZnSOD), by immunowestern blotting. A second set of rats were submitted to an ischemia-reperfusion protocol on Langendorff isolated perfused heart. A global total ischemia was realized for 30 min followed or not by 10 min or 2h of reperfusion. During IR hearts were perfused or not, with L-NAME (100 µM) or BH₄ (50µM) 5 min before ischemia and 5 early minutes of reperfusion. Myocardial function was evaluated all over the procedure (+/- dPdt, LV Developed Pressure), and infarct size was measured at 2h of reperfusion by coloration of heart slices with triphenyl trerazolium chloride (TTC). Coronary effluents were collected to evaluate NO metabolites (nitrites and nitrates) (Griess method). Biochemical measurements were done after 30min of ischemia or 10 min after post-ischemic reperfusion. eNOS expression, eNOS phosphorylation (Ser₁₁₇⁷), eNOS dimer/monomer ratio, MDA and nitrotyrosine were evaluated by immunowestern blotting or dot blot method.

Results and discussion

In accordance with scientific literature, exercise training reduced heart vulnerability to IR, characterized by a better functional recovery at reperfusion and a lower infarct size. Moreover, measurement of MDA at 10 minutes of reperfusion corroborates theses results demonstrating a reduced oxidative stress in Ex rats when compared to Sed rats. Exercise-induced cardioprotective phenotype was characterized, before IR, with increased eNOS phosphorylation at Ser₁₁₇⁷, without any changes regarding eNOS expression, dimer/monomer ratio, GCH-1 and Cu-ZnSOD expressions. In addition, no detectable difference of NO production was observed in coronary effluents between Sed and Ex rats. A first major result of the present work was that the major role played by constitutive NOS in such a cardioprotective phenotype was confirmed. Indeed in presence of L-NAME exercise-induced cardioprotection was blunted in Ex rats. Therefore, considering implication of eNOS in cardioprotection, we investigated whether preconditioning by exercise modulated eNOS dimer/monomer ratio during IR. The major result of the present work was that eNOS dimer/monomer ratio was significantly reduced in Ex rats compared to Sed rats from ischemia to early reperfusion. In addition, this increased monomerization of eNOS in Ex rats was associated with lower NO production during first minutes of reperfusion, followed by a normalization at 15 min. To test whether such an eNOS monomerization could contribute to protect the heart against IR injuries, we then perfused the myocardium during IR with BH₄. This pharmacological strategy significantly increased eNOS dimer/monomer ratio in Ex rats. The second major result of this study is that, in presence of BH₄ during IR and then, when eNOS dimer/monomer was normalized, a lack of exercise-induced cardioprotection was reported in Ex rats. Indeed, eNOS dimerization with BH₄ in Ex rats was associated with a reduced post-ischemic functional recovery and increased infarct size. These results are not in accordance with numerous studies reporting the beneficial effects of BH₄ supplementation on myocardial IR injury (Dumitrescu, Biondi et al. 2007; Masano, Kawashima et al. 2008) or various oxidative stress pathologies (Shinozaki, Nishio et al. 2005; Moens, Takimoto et al. 2008). However, to our knowledge, no study investigated the effect of BH₄ in interaction with exercise training preconditioning. In accordance with our work, Csonka et al. (Csonka, Szilvassy et al. 1999) and Ryou et al. (Ryou, Sun et al. 2008), reported in models of ischemic preconditioning a reduced myocardial sensitivity to IR associated with a reduced NO synthesis at reperfusion. Indeed, during post-ischemic reperfusion, the colocalization between coupled and uncoupled eNOS would promote ONOO⁻ formation, which is recognized as one of the most important cause of reperfusion injury. Therefore switching eNOS from dimer to monomer state and then form NO synthase to O₂⁻ synthase, could i) reduced ONOO⁻ formation, which could contribute to reduced cyto-toxicity related to nitrated oxygenated reactive species (Zweier, Fertmann et al. 2001; Levrand, Vannay-Bouchiche et al. 2006); and ii) promoted O₂⁻ synthesis., which could promote heart post-conditioning (Sanada, Komuro et al. 2011). It is then of note, that in the present work, lower nitrotyrosination of myocardial
proteins (170-72kda) was observed in Ex rats at early reperfusion, suggesting then lower formation of peroxynitrite in Ex rats compared to Sed ones.

To conclude, our study is the first to investigate involvement of eNOS in exercise-induced myocardial cardioprotection. Our major finding is that exercise-induced cardioprotection requires eNOS uncoupling.


Fig.1
Figure legends

Figure 1: Effects of exercise training and BH₄ supplementation on IR-induced cellular death. Representative sections of infarct size of rat hearts stained with triphenyltetrazolium chloride (TTC) after 30 min of global total ischemia and 120 min of reperfusion from isolated heart experiments in each experimental group. Infarct sizes expressed as percentage of left ventricular section. Data are presented as mean ± S.E.M., * p<0.05 Sed vs Ex, § p<0.05 Ex vs Ex + BH₄).

Figure 2: Effects of exercise training and BH₄ supplementation on eNOS dimerization. Expression of eNOS dimer/monomer ratio evaluated by immunoblotting after 30 min of global total ischemia and 10 min of reperfusion from isolated heart experiments in each experimental group. Data are presented as mean ± S.E.M., * p<0.05 Sed vs Ex, § p<0.05 Ex vs Ex + BH₄).