

EVIDENCE FOR A CEILING OF CARDIOPROTECTION WITH  
A NO DONOR-INDUCED DELAYED PRECONDITIONING IN RABBITS.

by

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## RUNNING TITLE PAGE

### CEILING OF PROTECTION WITH DELAYED PRECONDITIONING

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#### ***Words counts:***

Number of text pages : 15  
Number of tables : 1  
Number of figures : 3  
Number of references : 16  
Number of words in the Abstract : 231  
Number of words in the Introduction : 229  
Number of words in the Discussion : 874

#### ***Nonstandard abbreviations used in the paper :***

CAO, coronary artery occlusion  
ECG, electrocardiogram  
NO, Nitric oxide  
SNAP, S-nitroso-N-acetylpenicillamine  
TTC, triphenyltetrazolium chloride

**Recommended section : Cardiovascular**

## ABSTRACT

Although extensive attention has been devoted to the window of preconditioning, only few studies investigated the efficacy of preconditioning against ischemia with increasing durations. To date, a "ceiling of protection" has been demonstrated to occur with early preconditioning but nothing is known about delayed preconditioning. Accordingly, the efficacy of a NO-donor -induced delayed preconditioning was tested against ischemic insults of increasing duration. Accordingly, sixty-five rabbits received a 75-min intravenous infusion of either saline (Control group), or a NO-donor (S-nitroso-N-acetylpenicillamine) at 3  $\mu\text{g}/\text{kg}/\text{min}$  (SNAP-3 group) or 30  $\mu\text{g}/\text{kg}/\text{min}$  (SNAP-30 group). Twenty-four hours later, rabbits randomly underwent either a 15, a 20 or a 30-min coronary artery occlusion (CAO). Infarct size was assessed after 72 h-reperfusion (TTC staining, % of the area at risk). After 15-min CAO, both SNAP-3 and SNAP-30 reduced infarct size as compared to Control ( $10\pm 3\%$ ,  $5\pm 1\%$  vs  $29\pm 8\%$ , respectively,  $p < 0.05$ ). After 20-min CAO, significant cardioprotection was only observed with SNAP-30 ( $29\pm 4\%$ ,  $21\pm 6\%$  vs  $36\pm 2\%$  for SNAP-3, SNAP-30 vs Control, respectively). After 30-min CAO, both SNAP-3 and SNAP-30 failed to reduce infarct size ( $48\pm 2\%$ ,  $50\pm 5\%$  vs  $50\pm 4\%$  for SNAP-3, SNAP-30 vs Control, respectively). In conclusion, this study demonstrates a dose-related "ceiling of protection" with delayed preconditioning induced by a NO-donor. It supports that delayed preconditioning might exert its maximal beneficial effect with early reperfusion and this finding supports the necessary use of different durations of ischemia when investigating cardioprotective strategies.

Ischemic and pharmacological preconditioning are characterized by an early and a delayed phase of preconditioning (Murry et al., 1986; Marber et al., 1993). To date, the window of cardioprotection, *i.e.* the time-interval between the preconditioning stimulus and the final sustained ischemia, has received extensive attention (Kloner and Jennings, 2001). The mechanism of this cardioprotection has been extensively investigated and appears to be both complex and highly multifactorial (Bolli, 2000; Napoli et al., 2000; Schulz et al., 2001). However, only few studies have demonstrated that the efficacy of early preconditioning progressively disappears with increasing durations of ischemia, thus characterizing a "ceiling of protection" (Murry et al., 1986; Gumina et al., 1999). To date, this issue has never been investigated with delayed preconditioning.

Nitroglycerin and nitric oxide (NO)-donors such as S-nitroso-N-acetylpenicillamine (SNAP) have demonstrated their efficacy at inducing delayed preconditioning both experimentally (Takano et al., 1998; Hill et al., 2001) and clinically (Leesar et al., 2001). This cardioprotection has been reported to involve the generation of oxidant species, the activation of protein kinase C and nuclear factor-kappa B as well as the induction of iNOS activity (Bolli, 2000). Accordingly and taking into account our background with SNAP (Lellouche et al., 2002), we decided to investigate the ceiling of protection with this NO-donor. Myocardial infarct size was measured after increasing durations of coronary artery occlusion followed by a long duration of reperfusion.

## METHODS

The experiments were performed in accordance with the official regulations edicted by the French Ministry of Agriculture (Agreement # A94-043-12).

### *Animal surgery*

Male New Zealand rabbits (2-2.5 kg) were anesthetized with pentobarbital sodium (20-30 mg/kg, i.v.). They were intubated and mechanically ventilated with 100% oxygen (ventilation rate : 25 breaths per minute ; tidal volume : 25 ml). A catheter was positioned in the rabbit's ear marginal artery for arterial pressure measurement (Statham P23ID strain gauge, Statham Instruments, Oxnard, CA, USA). An external electrocardiogram (ECG) was also recorded. A left thoracotomy was performed at the fourth intercostal space under sterile conditions. The pericardium was opened and a 4/0 Prolene suture was passed beneath a major branch of the left coronary artery. The ends of the ligature were passed through a short segment of propylene tubing to form a snare. Regional myocardial ischemia was induced by pulling the snare through the tubing. Ischemia was confirmed by the presence of regional modifications of the myocardial surface and by the occurrence of ST segment deviation of the ECG. In all animals, a coronary artery occlusion (CAO) was performed and the snare was released. The chest was then closed in layers and a small tube was left in the thorax to evacuate air and fluids after surgery. All rabbits underwent 72 h of coronary artery reperfusion.

### *Measurement of risk area and infarct size*

After completion of reperfusion, animals received heparin and sodium pentobarbital (50 mg/kg, i.v.). Potassium chloride was then administered i.v. to induce cardiac arrest. The

hearts were excised. The ascending aorta was cannulated and perfused (120 mmHg) retrogradely with saline followed by Evans blue (1%) after ligation of the previously occluded artery. The left ventricle was cut into 8-10 slices. These slices were weighed and incubated with 1 % triphenyltetrazolium chloride (TTC, Sigma, Poole, U.K.) in a pH 7.4 buffer during 15 minutes at 37°C. Slices were overnight fixed in 10% formaldehyde and then photographed with a digital camera. Using a computerized planimetric program (Scion Image, Scion Corporation, Frederick, MD, USA), the area at risk and the infarcted zones were quantified. The area at risk was identified as the non-blue region and was expressed as a percentage of the left ventricle weight. Infarcted area was identified as the TTC negative zone and was expressed as a percentage of the area at risk.

#### *Experimental protocol*

The animals were randomized and the protocol was realized during two consecutive days, *i.e.*, 24 h apart as illustrated in Figure 1. On day 1, rabbits were divided into three groups receiving a 75-min intravenous infusion of either saline (Control group), S-nitroso-N-acetylpenicillamine at 3 µg/kg/min (SNAP-3 group) or S-nitroso-N-acetylpenicillamine at 30 µg/kg/min (SNAP-30 group). These doses of SNAP were chosen on the basis of a preliminary study showing that 3 µg/kg/min did not induce any hemodynamic effect whereas 30 µg/kg/min significantly decreased mean arterial pressure (-14% from 89±3 mmHg). On day 2, all rabbits underwent either a 15, a 20 or a 30-min CAO followed by 72h reperfusion.

#### *Statistical analysis*

Data are reported as mean ± SEM. Comparisons were made using ANOVA followed by post-hoc Fisher's PLSD test if necessary. Significant differences were determined as  $p < 0.05$ .

## RESULTS

Sixty-five rabbits successfully underwent the whole protocol: (i) 7 Control, 6 SNAP-3 and 6 SNAP-30 with 15-min CAO, (ii) 10 Control, 7 SNAP-3 and 7 SNAP-30 with 20-min CAO, and (iii) 8 Control, 8 SNAP-3 and 6 SNAP-30 with 30-min CAO.

### *Hemodynamic*

On day 1, baseline values of heart rate and mean arterial pressure were not significantly different between groups (heart rate: 202±8, 196±12, and 197±5 beats/min and mean arterial pressure: 99±4, 95±4, and 97±3 mmHg for all Control, SNAP-3 and SNAP-30, respectively). Infusion of saline and SNAP at 3 µg/kg/min did not significantly affect these parameters (heart rate: 208±9 beats/min and mean arterial pressure: 98±3 mmHg for SNAP-3). SNAP at 30 µg/kg/min reduced mean arterial pressure by 15% and increased heart rate by 25% (82±4 mmHg and 247±7 beats/min,  $p < 0.05$ , respectively). On day 2, these parameters were not significantly different between all groups at baseline, during CAO and reperfusion (data not shown).

### *Infarct sizes*

Sizes of area at risk were similar among groups (Table I). After 15-min CAO, infarct sizes were significantly reduced by SNAP-3 and by SNAP-30 as compared to Control (figure 2). After 20-min CAO, SNAP-3 failed to reduce infarct size whereas SNAP-30 still exerted beneficial effects. After 30-min CAO, infarct sizes were similar among groups. As illustrated in Figure 3, the intensity of cardioprotection (*i.e.*, reduction of mean infarct size vs Control) was plotted against the duration of CAO. Interestingly, SNAP-30 provided a near maximal cardioprotection after 15-min CAO (-84%). However, a ceiling of protection was observed with SNAP-30 after 30-min CAO and also with SNAP-3 after 20-min CAO.

## DISCUSSION

To our knowledge, this is the first study to demonstrate a "ceiling of protection" with a pharmacological delayed preconditioning against myocardial infarction. Indeed, administration of a NO-donor (SNAP) exerted a powerful delayed cardioprotection after a short but not after a prolonged coronary occlusion. Importantly, these results were observed after 72h of reperfusion, excluding any confusion due to insufficient durations of reperfusion (Miki et al., 1999; Tissier et al., 2002).

In literature, the evaluation of the limiting effect of any procedure on infarct size is usually performed using a single duration of CAO. However, Gumina *et al.* (1999) demonstrated in dogs that two different cardioprotective procedures (i.e., a  $\text{Na}^+/\text{H}^+$  exchange inhibitor and early preconditioning) might be equipotent to reduce infarct size after a 60-min CAO but not after 90-min CAO. Thus, the use of a single CAO duration does not provide complete information about the cardioprotective potency of a drug or a preconditioning maneuver. Accordingly, we investigated SNAP-induced delayed preconditioning with 3 different durations of CAO in the present study. Although this pharmacological strategy was able to exert a potent cardioprotective effect after short periods of ischemia, the magnitude of cardioprotection significantly diminished with increasing durations of CAO and hence with subsequent increasing in infarct size. This study clearly demonstrates a "ceiling of cardioprotection" with delayed preconditioning as it was demonstrated with early ischemic preconditioning (Gumina et al., 1999). Interestingly, the time to reach this "ceiling of protection" with SNAP was dose-dependent and therefore related to the intensity of the preconditioning stimulus. One can argue that higher doses of SNAP would have further delay the time to reach the "ceiling point" but regarding the potent and undesirable hypotensive effect of higher doses than 30  $\mu\text{g}/\text{kg}/\text{min}$  of SNAP, it was difficult to test this hypothesis.



Many parameters such as anesthesia, the sedation protocol in conscious preparations (*e.g.*, use of diazepam), the size of area at risk (Ytrehus et al., 1994) and strains of animals (Bao et al., 2000) may modify the myocardial oxygen balance, the severity of ischemia and hence the susceptibility to infarction. It is thus interesting to speculate that different experimental conditions would lead to different patterns of "ceiling of protection". Indeed after 30-min CAO, our present and previous results (Lellouche et al., 2002) failed to demonstrate any protective effect of SNAP whereas others reported a significant reduction in infarct size in rabbits (Takano et al., 1998). However, the investigation of increasing durations of CAO allows to avoid discrepancies between studies and leads to the same final conclusion, *i.e.*, delayed preconditioning with SNAP is effective at reducing infarct size, even after 72h of reperfusion. Therefore, the use of a single duration of CAO is certainly one explanation to account for discrepancies observed between studies regarding the cardioprotective effects of a drug or of a preconditioning maneuver.

Importantly, it is well known that the prolongation of ischemia is responsible for an increase in infarct size that will tend to a maximal value, *i.e.*, about 80-90% of the area at risk in rabbits (Miura et al., 1989). Preventive cardioprotective strategies tend to be inefficient when this maximal value is reached, *e.g.*, after 60 to 90-min CAO in native collateral deficient species such as rabbit (Miura et al., 1989). In contrast, in the present study, the loss of protection conferred by delayed preconditioning with SNAP is different in its mechanism. Indeed, the "ceiling of protection" appears with infarct sizes averaging 50% of the area at risk, *i.e.*, far from the maximal possible infarct size (Miura et al., 1989). In other words, delayed preconditioning with SNAP cannot provide in all cases additional salvage of myocardial tissue to the beneficial effects of reperfusion, *i.e.*, it will be beneficial only with early revascularisation. Consequently, it becomes important not only to define the ability of a drug

to reduce infarct size but also to characterize its "ceiling of protection", *i.e.*, the duration of ischemia and the infarct size at which protection is lost.

Although we did not specifically investigate this issue, it is tempting to speculate that the concept of "ceiling of protection" could be extended to other stimuli known to induce delayed preconditioning, *e.g.*, brief ischemia or adenosine, which partially share common signaling pathways with NO-donors (Bolli, 2000). Indeed, both ischemic and pharmacological delayed preconditioning with an adenosine A<sub>1</sub>-receptor agonist were unable to reduce infarct size after 30min CAO and 72 h reperfusion in rabbits (Miki et al., 1999; Lellouche et al., 2002; Tissier et al., 2002). These data suggest once again that a ceiling of protection was reached but additional studies are required to definitely extend this concept to all kind of delayed preconditioning. Investigation of the mechanisms responsible for a "ceiling of protection" were beyond the scope of this study. One could speculate, however, that differential interactions exist among the numerous mediators of cardioprotection and those of myocardial injury with varying durations of ischemia.

In conclusion, our results demonstrate that delayed preconditioning induced by the NO-donor, SNAP, reaches a "ceiling of protection" against myocardial infarction in rabbits. Moreover, our results support the necessary use of different durations of ischemia when investigating cardioprotective strategies. This finding has also potential important clinical implications supporting that delayed preconditioning might exert its optimal beneficial effect mainly with early revascularization.

## ACKNOWLEDGMENTS

The authors are greatly indebted to Alain Bizé and Stéphane Bloquet for their excellent technical support.

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## FOOTNOTES

### *Financial support :*

This study was supported by grants from the Fondation de l'Avenir # ET2-293 and from BQR 2002 (Université Paris-Sud).

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## LEGENDS FOR FIGURES

### ***Figure 1***

Experimental protocol

(DPC: delayed preconditioning; CAO: coronary artery occlusion; CAR: coronary artery reperfusion; SNAP-3: delayed preconditioning with S-nitroso-N-acetylpenicillamine at 3  $\mu\text{g}/\text{kg}/\text{min}$  during 75 min; SNAP-30: delayed preconditioning with S-nitroso-N-acetylpenicillamine at 30  $\mu\text{g}/\text{kg}/\text{min}$  during 75 min).

### ***Figure 2***

Infarct sizes (expressed as percentage of the area at risk) measured after increasing durations of coronary artery occlusion in Control, SNAP-3 (delayed preconditioning with S-nitroso-N-acetylpenicillamine at 3  $\mu\text{g}/\text{kg}/\text{min}$  during 75 min) and SNAP-30 (delayed preconditioning with S-nitroso-N-acetylpenicillamine at 3  $\mu\text{g}/\text{kg}/\text{min}$  during 75 min).

### ***Figure 3***

Percentage of cardioprotection afforded by SNAP (*i.e.*, percentage of the reduction in mean infarct sizes as compared to Control) is plotted against the duration of coronary artery occlusion (min).

**TABLE 1** : Left ventricular weight (expressed in grammes) and area at risk (expressed as percentage of the left ventricle weight) in the different groups

		<i>n</i>	<i>LV (g)</i>	<i>AAR (% LV)</i>
<i>CAO 15 min</i>	Control	7	4.5 ± 0.4	35 ± 4
	SNAP-3	6	4.4 ± 0.2	31 ± 5
	SNAP-30	6	3.9 ± 0.2	37 ± 4
<i>CAO 20 min</i>	Control	10	4.1 ± 0.3	33 ± 3
	SNAP-3	7	4.1 ± 0.1	39 ± 5
	SNAP-30	7	4.1 ± 0.3	36 ± 3
<i>CAO 30 min</i>	Control	8	4.5 ± 0.2	38 ± 3
	SNAP-3	8	4.3 ± 0.1	34 ± 4
	SNAP-30	6	4.3 ± 0.2	34 ± 2

Values are mean ± S.E.M.

CAO: coronary artery occlusion; LV: left ventricle; AAR: area at risk

SNAP-3: delayed preconditioning with S-nitroso-N-acetylpenicillamine at 3 µg/kg/min during 75 min

SNAP-30: delayed preconditioning with S-nitroso-N-acetylpenicillamine at 30 µg/kg/min during 75 min







