The Nitric Oxide-Releasing Naproxen Derivative Displays Cardioprotection in Perfused Rabbit Heart Submitted to Ischemia-Reperfusion

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ABSTRACT

In this study, the pharmacological activity of HCT-3012 ([S]-6-methoxy-α-methyl-2-naphtaleneacetic acid 4-(nitrooxy)butyl ester), a nitric oxide (NO)-releasing derivative of naproxen, was compared with that of naproxen in a model of acute ischemia (40 min) and reperfusion (20 min) of the rabbit heart. HTC-3012 (3–100 μM), in spite of inhibition of 6-keto-prostaglandin F1α generation by the cardiac tissues, brought about a dose-dependent normalization of coronary perfusion pressure, associated with a reduction of ventricular contracture during ischemia with remarkable improvement of left ventricular developed pressure at reperfusion. These beneficial effects were accompanied by a substantial release of nitrite/nitrate in the heart perfusates, indicating that NO has been released by HCT-3012 and donated to the cardiac tissue. These events were paralleled by a significant reduction of creatine kinase activity in heart perfusates during reperfusion. Naproxen (10–100 μM) aggravated the myocardial damage in ischemic reperfused hearts, severely depressing the postischemic ventricular dysfunction. Perfusion of the heart with Nω-monomethyl-L-arginine (10 μM) caused a marked aggravation of myocardial damage of the reperfused hearts, and this effect was dose-dependently prevented by HCT-3012 but not by naproxen. The results of the present experiments clearly indicate that HCT-3012, by donating NO, displays a noticeable anti-ischemic effect in reperfused ischemic rabbit hearts. The safer gastrointestinal profile of HCT-3012 and its ability to control experimental hypertension, suggest that this compound may have therapeutic potential in cardiovascular disease, namely in the prevention of myocardial ischemic events, and may represent a better alternative to conventional nonsteroidal anti-inflammatory drugs.

In the last decade, an approach that aims to improve organ tolerability of nonsteroidal anti-inflammatory drugs (NSAIDs) and involving derivatization of these compounds to incorporate nitric oxide (NO)-releasing moiety, has gained the attention of the scientific community (Wallace et al., 1997; Fiorucci et al., 2001). This new class of drugs, known as “NO-NSAIDs”, discloses similar or even ameliorated anti-inflammatory and analgesic activities compared with parent compounds (Wallace et al., 1994, 1995; Davies et al., 1997). Among them, the NO-releasing derivative of naproxen (HTC-3012; [S]-6-methoxy-α-methyl-2-naphtaleneacetic acid 4-(nitrooxy) butyl ester) exhibits anti-inflammatory activity of comparable potency to naproxen, analgesic activity superior to naproxen, and greatly reduced toxicity in the gastrointestinal tract (Davies et al., 1997; Muscarà et al., 1998; Cicala et al., 2000). Moreover, HTC-3012 has been reported to reduce blood pressure in rats with Nω-nitro-L-arginine methyl ester-induced hypertension (Muscarà et al., 1998) likely via hypersensitivity of vascular smooth muscle to the exogenous NO that develops when NO-synthase is chronically suppressed (Henrion et al., 1996). The antihypertensive property of HTC-3012 has also been demonstrated in the “two kidney, one clip” renovascular hypertension model in rats (Muscarà et al., 2000). In this regard, it has been speculated that the reduction of blood pressure determined by this compound was not simply due to the vasodilatory action of the released NO, but to attenuation of the sympathetic control of blood vessel tone and alteration in the responsiveness of the vasculature to endogenous pressure agents. Positive results have already been shown with another

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; NO, nitric oxide; HCT-3012, (S)-6-methoxy-α-methyl-2-naphtaleneacetic acid 4-(nitrooxy)butyl ester; KHS, Krebs-Henseleit solution; CPP, coronary perfusion pressure; LVP, left ventricular pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVEDV, left ventricular developed pressure; CK, creatine kinase; PGI2, prostacyclin; NOx, nitrite/nitrate; 6-keto-PGF1α, 6-keto-prostaglandin F1α; l-NMMA, Nω-monomethyl-L-arginine; NCX 4016, 2-acetoxy-benzoate 2-(1-nitroxy-methyl)-phenyl ester; AUC, area under the curve.
NO-NSAID, the nitroderivative of aspirin, NCX 4016, demonstrating a relevant cardioprotection likely mediated by NO donation. In fact, this compound minimized cardiac mechanical abnormalities induced by ischemia in perfused rabbit hearts and remarkably reduced the mortality rate in rabbits with permanent ligation of anterior coronary artery (Rossoni et al., 2000). This nitroderivative of aspirin was also very active in diminishing infarct size caused by regional myocardial ischemia and reperfusion in anesthetized rats (Rossoni et al., 2001) and pigs (Wainwright et al., 2002).

All this information prompted us to investigate the possible protecting activity of HCT-3012 in a model of perfused rabbit heart subjected to global ischemia and reperfusion. The results of these studies could be of potential clinical interest and have come at the right moment because the discussion around no reduction of cardiovascular risk with NSAIDs is strongly elevated (Cleland, 2002; Ray et al., 2002; Vane, 2002).

### Materials and Methods

**Animals.** Male New Zealand White rabbits (BMG-Allevamento, Cividate al Piano, BG, Italy), weighing 1.8 to 2.0 kg, were used. The animals were housed in a conditioned environment (22 ± 1°C, 55 ± 5% relative humidity, 12-h light/dark cycle) and were given free access to food and tap water. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, revised 1996).

**Perfusion of Hearts.** Perfusion of rabbit heart was performed as described previously (Rossoni et al., 2000). In brief, the rabbits were anesthetized with sodium pentobarbital (60 mg/kg) given by intravenous injection. The chest was opened, and the heart was rapidly excised and placed in cold (4°C) Krebs-Henseleit solution (KHS) of the following composition: 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.6 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 11.5 mM glucose. The heart was quickly removed within 2 min after thoracotomy and mounted on the experimental setup. The heart was perfused retrogradely at 20 ml/min (Minipuls-3 peristaltic pump; Gilson, Villiers-Le Bel, France) via the aorta with KHS, which was maintained at 37°C and aerated with 95% O₂ + 5% CO₂ to maintain normal pH, pO₂, and pCO₂ parameters. Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were measured with two HP-1280C pressure transducers (Hewlett Packard, Waltham, MA) connected to a Hewlett Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter, with a small latex balloon on the tip (4; Hugo Sachs Elektronik, March-Hugstetten, Germany), inserted into the left ventricular cavity through the mitral valve opening. The volume of the balloon was adjusted to give a peak left ventricular systolic pressure (LVSP) of 95 to 100 mm Hg with a left ventricular end-diastolic pressure (LVEDP) of 5 to 6 mm Hg. Hearts that could not achieve this level of contractile performance (7–8% of the hearts) were excluded. Left ventricular developed pressure (LVdP) was also evaluated.

**Ischemia and Reperfusion.** After equilibration of 15 min, hearts were paced at 180 beats/min with an electrical stimulator (S-88; Grass Instruments, Quincy, MA) via two silver electrodes attached to the right atrium, and an additional 30 min of perfusion was carried out (preischemic period). Ischemia was induced by reducing the flow rate from 20 to 1 ml/min for 40 min (ischemic period). Normal flow rate (20 ml/min) was then restored, and the perfusion was continued for another 20 min (reperfusion period). Throughout the experiment, a thermoregulated chamber maintained the heart temperature at 37°C to avoid hypothermia-induced cardioprotection.

![Fig. 1. Effect of HCT-3012 and naproxen on LVEDP in paced isovolumic left rabbit heart preparations subjected to low-flow ischemia and reperfusion. Top, compounds were infused for 20 min before reduction of flow (top). Bottom, AUC for all concentrations of each drug used. +, P < 0.05; ++, P < 0.01; +++ P < 0.001 versus the vehicle-treated group (white column). Each point/bar represent the mean ± S.E.M. of six to eight experiments.](image-url)
The total duration for each experiment did not exceed 2 h, during which time the experimental preparation was stable.

After preliminary experiments, different molar concentrations of the compounds under investigation were selected and tested in groups of six to eight hearts each. In particular, HCT-3012 (3, 10, 30, and 100 μM) or naproxen (10, 30, and 100 μM) were perfused through the hearts for a period of 20 min before reduction of coronary flow.

Creatine Kinase (CK) Activity in Heart Perfusates. The perfusate, eluted from the rabbit heart during preischemic and reperfusion periods, was collected in an ice-cooled beaker as 2.5-min samples. Each sample was used for the determination of CK activity according to the method of Bergmeyer et al. (1970). The total amount of this enzyme activity was measured spectrophotometrically (λ=410; PerkinElmer Italia, Monza, MI, Italy) at 37°C by using a commercial assay kit. Data are expressed as milliunits per gram of wet tissue per minute.

Prostacyclin (PGI₂) and Nitrite/Nitrate (NOx) Determinations in Heart Perfusates. PGI₂ and NOx were measured directly in the coronary effluent collected in an ice-cooled beaker for 1 min immediately before ischemia and during the first 10 min of reperfusion. PGI₂ was determined as its stable metabolite 6-keto-prostaglandin F₁₅ (6-keto-PGF₁₅) according to the enzyme-linked immunosorbent assay (ELISA) method of Pradellas et al. (1985), whereas NOx levels were analyzed by using a fluorometric assay kit (detection limit, 10 pmol/ml) (Misko et al., 1993). PGI₂ and NOx were assayed in duplicate, and the results were expressed as nanomoles per minute.

Statistical Analysis. Each value represents the mean ± S.E.M. of six to eight experiments.

Results

Ischemia-Reperfusion in Isolated Rabbit Heart. When the rate of perfusion of electrically paced isovolumic left rabbit heart preparations was reduced from 20 to 1 ml/min for 40 min, LVEDP values increased progressively indicating that, after standstill, an ischemic process was occurring. In fact, at reperfusion ventricular function was impaired, LVEDP values being significantly reduced and CPP considerably increased above baseline (Figs. 1-3).

When the hearts were perfused for 20 min with graded concentrations of HCT-3012 (3, 10, 30, and 100 μM) in the preischemic period, a dose-dependent myocardial protection against mechanical changes due to ischemia-reperfusion was recorded. In fact, the characteristic ventricular contracture observed during the 40 min of ischemia was reduced, and this event favored a better recovery of LVEDP at reperfusion (Figs. 1 and 3). At the same time, CPP was diminished as a function of the dose of HCT-3012 used (Fig. 2).

In clear contrast with the results reported above, the perfusion of the hearts with naproxen (10, 30, and 100 μM) produced severe worsening of myocardial ischemic damage. In fact, the LVEDP values were significantly increased compared with those of vehicle-treated preparations, and this phenomenon was associated with a marked depression of LVEDP and an increase in CPP at reperfusion (Figs. 1–3). Naproxen did not alter CPP, LVEDP, and LVEDP during the preischemic period. The lack of any effect on CPP by naproxen during the preischemic period indicates that probably the coronary bed in this preparation is already maximally dilated or lack of PGI₂ involvement.

![Fig. 2. Effect of HCT-3012 and naproxen on CPP in paced isovolumic left rabbit heart preparations subjected to low-flow ischemia and reperfusion. Top, compounds were infused for 20 min before reduction of flow. Bottom, AUC for all concentrations of each drug used. * P < 0.05; ** P < 0.01; *** P < 0.001 versus the vehicle-treated group (white column). Each point/bar represent the mean ± S.E.M. of six to eight experiments.](https://jpet.aspetjournals.org/doi/10.1124/jpet.187.1.10021)
CK Activity in Heart Perfusates. CK, an indicator of myocardial damage, was determined in the coronary effluent collected from each heart in a 2.5-min sample during preischemic and reperfusion periods. As shown in Fig. 4, there were no differences between the various groups of hearts in CK release during the preischemic period. However, during 20-min reperfusion, CK activities measured in the vehicle-treated group were 5.4-fold higher (P < 0.001) than those found in the preischemic period (75±5 mU/g wt/min) (Fig. 4).

Perfusion for 20 min before ischemia with HCT-3012 (3–100 μM) reduced CK release in a concentration-dependent manner at reperfusion compared with vehicle-treated hearts (Fig. 4).

At variance with HCT-3012, the severity of postischemic ventricular dysfunction caused by naproxen was associated with a marked increase of CK activity in heart effluents compared to vehicle-treated hearts (Fig. 4).

PGI2 Release in Heart Perfusates. It is well known that PGI2 is the major eicosanoid produced by jeopardized myocardium (Van Bilsen et al., 1989) and that the rate of formation of this lipidic material increases particularly during the first 5–10 min of reperfusion declining rapidly thereafter (Berti et al., 1988; Engels et al., 1990). In the present study, in vehicle-treated hearts the generation of 6-keto-PGF1α, during reperfusion was enhanced 3.5-fold (P < 0.001) compared with the preischemic period (2.88±0.32 ng/min) (Table 1).

When the hearts were perfused with HCT-3012 or naproxen, the release of 6-keto-PGF1α was inhibited in a concentration-dependent manner in both preischemic and reperfusion periods. In particular, the inhibitory effect of naproxen on 6-keto-PGF1α release was observed at a concentration 3-fold lower than that required for obtaining a similar inhibition by HCT-3012 (Table 1).

NOx Release in Heart Perfusates. The results of NOx concentrations in heart perfusates are shown in Table 2. In vehicle-treated hearts, the NOx levels measured before ischemia were lower than the levels found during reperfusion. However, when the hearts were perfused with HCT-3012 or naproxen, the release of NOx was inhibited in a concentration-dependent manner.
did not affect the basal release of NOx generation (Table 2). NOx in both periods. Naproxen, at all concentrations used, data reported in the reperfusion period are significantly different (P < 0.001) from that obtained in the preischemic period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOx Preischemia</th>
<th>NOx Reperfusion*</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>5.72 ± 0.48</td>
<td>2.12 ± 0.27</td>
</tr>
<tr>
<td>HCT-3012, 3 μM</td>
<td>6.14 ± 0.57</td>
<td>4.35 ± 0.15</td>
</tr>
<tr>
<td>HCT-3012, 10 μM</td>
<td>9.01 ± 0.74**</td>
<td>3.21 ± 0.44*</td>
</tr>
<tr>
<td>HCT-3012, 30 μM</td>
<td>9.94 ± 0.68***</td>
<td>4.01 ± 0.35**</td>
</tr>
<tr>
<td>HCT-3012, 100 μM</td>
<td>11.69 ± 1.03***</td>
<td>4.72 ± 0.53***</td>
</tr>
<tr>
<td>Naproxen, 10 μM</td>
<td>4.97 ± 0.38</td>
<td>2.55 ± 0.33</td>
</tr>
<tr>
<td>Naproxen, 30 μM</td>
<td>6.15 ± 0.52</td>
<td>1.92 ± 0.24</td>
</tr>
<tr>
<td>Naproxen, 100 μM</td>
<td>5.93 ± 0.35</td>
<td>2.30 ± 0.15</td>
</tr>
</tbody>
</table>

* Data refer to the first 10 min of reperfusion.
** P < 0.05 and *** P < 0.001 versus the vehicle-treated hearts.

emia (5.72 ± 0.48 nmol/min) markedly decrease during reperfusion (2.12 ± 0.17 nmol/min; P < 0.001).

During the preischemic and reperfusion periods, the perfusion of the hearts with HCT-3012 (30–100 μM) caused a concentration-dependent increase of NOx in the perfusate. This event was particularly marked at the concentration of 100 μM where HCT-3012 doubled the rate of formation of NOx in both periods. Naproxen, at all concentrations used, did not affect the basal release of NOx generation (Table 2).

## NO-Synthase Inhibition in Rabbit Heart Subjected to Ischemia-Reperfusion

Perfusion of the hearts with L-NMMA (10 μM) for 10 min before flow reduction exacerbated ventricular dysfunction compared with vehicle-treated preparations. In fact, at the end of the ischemic period, LVEDP increased from 4.7 ± 0.3 to 102 ± 7 mm Hg (1.7-fold higher than that of the corresponding value in vehicle-treated hearts; P < 0.001). After 20 min of reperfusion these values were still in the range of 95 ± 6 mm Hg (Fig. 5), and the mechanical activity was severely impaired and associated with cardiac rhythm disturbance (data not shown).

Furthermore, during L-NMMA treatment, the CPP rose from 50 ± 4 to 105 ± 8 mm Hg (P < 0.001), and at the end of reperfusion this value was in the range of 136 ± 9 mm Hg; that is, 1.5-fold higher (P < 0.001) than that observed in vehicle-treated hearts (Fig. 6 and 7). These mechanical changes of the hearts resulted in severely depressed left ventricular function during reperfusion (reduced compliance). In fact, at the end of this period LVDevP was confined to 8.7 ± 2 mm Hg (68% reduction; P < 0.001 versus preischemic values) (data not shown).

Treatment of the hearts with HCT-3012 (10–100 μM) dose-dependently reduced the ventricular contracture caused by the NO-synthase inhibition during the ischemic period. Consequently, in these treated hearts a dose-dependent amelioration of mechanical activity with regular electrical pacing was recorded at reperfusion (data not shown).

On the contrary, treatment of the hearts with naproxen (10–100 μM) did not interfere with worsening heart mechan-

**Fig. 5.** L-NMMA aggravates LVEDP in paced isovolumic left rabbit heart preparations subjected to low-flow ischemia and reperfusion (top). HCT-3012 or L-arginine, but not naproxen, counteract the deleterious effect induced by L-NMMA on LVEDP. The compounds under investigation were given through the hearts for 20 min followed by 10-min infusion with L-NMMA. Bottom, AUC for all concentrations of each drug used. All the L-NMMA-treated groups were significantly different (P < 0.01 to P < 0.001) versus the vehicle-treated group (white column); ***, P < 0.001; ***, P < 0.001 versus the L-NMMA-treated group (black column). Each point/bar represent the mean ± S.E.M. of six experiments.
ics and CPP caused by l-NMMA during preischemic, ischemic, and reperfusion periods (Figs. 5–7).

Treatment of the hearts with l-arginine (100 μM) fully prevented the increase of LVEDP and CPP brought about by l-NMMA during preischemic ischemic and reperfusion periods (Figs. 5–7).

**Discussion**

In the present study, isovolumic left heart preparation perfused at low flow was used as a model of acute myocardial ischemia. In this model, the major determinants of myocardial performance, including cardiac frequency, ventricular size (muscle length), coronary flow, and composition of the perfusate, are under control. Moreover, changes in peak systolic LVdP/dt and LVEDP are used as indexes of cardiac contractility and diastolic elastic stiffness (compliance). Although the isolated perfused heart has certain limitations, this preparation is particularly useful for interventions that, in the intact organism, may act directly and/or indirectly on the heart (Henry et al., 1977).

The present results clearly demonstrate that the NO-releasing naproxen derivative, HCT-3012, exhibits a noticeable cardioprotective activity, preventing cardiac mechanical abnormalities caused by 40 min of global myocardial ischemia followed by 20-min reperfusion in perfused rabbit hearts. HCT-3012 reduces the increased values of LVEDP and CPP in a dose-related manner with a marked improvement of myocardial contractility at reperfusion. These beneficial effects were accompanied by a decrease of CK in cardiac perfusates, indicating that the loss of functional integrity of sarcolemma was diminished in spite of impairment of PGI2 formation by the cardiac endothelial cells.

This may imply that NO moiety released by HCT-3012 contributed in great part to the cardioprotective activity observed with this compound in ischemic reperfused rabbit hearts. Indeed, the direct determination of NOx concentrations in cardiac tissues or in heart perfusates is mandatory and, at the moment, this information is not available. However, indirect proof of this crucial point is provided by a dose-dependent increase of NOx concentrations determined in perfusates of the hearts treated with HTC-3012 during both preischemic and reperfusion periods. Furthermore, in the present experimental model the blockade of NO synthase with L-NMMA markedly increased the ventricular dysfunction at reperfusion, as shown by the highly elevated LVEDP and CPP values. These severe mechanical changes in the heart were inhibited by l-arginine and to a large extent by HCT-3012. In this set of experiments, naproxen was unable to control the negative effects of l-NMMA in the reperfused hearts. In similar experiments, it has recently been reported that HCT-3012, given orally for 4 weeks to rats, was very effective in preventing gastric damage and reducing hypertension, both of which are induced by Nω-nitro-l-arginine methyl ester (Muscarà et al., 1998).
It is worth stressing that naproxen, depending on the dose used, aggravated myocardial damage in reperfused hearts in the absence of L-NMMA. In this instance, LVDevP values were severely or significantly depressed by naproxen during reperfusion. These worsening effects of naproxen are very likely due to the removal of prostaglandins via cyclooxygenase impairment, an event that was not counterbalanced as in the case of HCT-3012 (NO donation). In this regard, it is well known that prostaglandin formation, namely PGI₂, represents a critical cytoprotective mechanism against the damage caused by ischemia (Ogilree et al., 1979; Berti et al., 1987, 1993). Indeed, the rate of PGI₂ formation in the ischemic reperfused rabbit heart has been shown to increase with the severity of the ischemic process (Berti et al., 1988). Stabilization of cardiac lysosomes provided by normal generation of PGI₂ is of paramount importance in the ischemic myocardium, because leakage of lysosomal enzymes (proteases and phospholipases) may contribute to irreversible damage of cardiomyocytes (Wildenthal et al., 1978).

Regarding the anti-ischemic effect of HCT-3012, the question of its action mechanism arises, principally in view of the findings showing that exogenous NO exerts a direct relaxant action on the myocardium independent of its vasodilatory activity and without compromising systolic function (Grocott-Mason et al., 1994; Kelley et al., 1996). Thus, it is tempting to speculate that the NO moiety donated by HCT-3012 to cardiac myocytes may have increased intracellular cyclic GMP and restricted the depletion of energy stores in ischemic cells promoting the dissociation of actin-ADP-myosin complexes (cross-bridges) and reducing ventricular stiffness (Henry et al., 1977).

In conclusion, the results obtained with the present experiments underline that the NO-releasing naproxen derivative exhibits a remarkable anti-ischemic action in reperfused rabbit hearts. Donation of NO to the cardiac tissues by HCT-3012 seems to be the main contributor to the cardioprotective activity of this compound. The convenient pharmacological profile in the gastrointestinal tract, and the ability to control elevated blood pressure will make NO-naproxen a safer alternative to standard NSAIDs in the treatment of cardiovascular disease, namely, in the prevention of myocardial ischemic events. At this point, it is interesting to emphasize that a recent epidemiological analysis failed to detect a reduction in myocardial infarction among patients prescribed naproxen with a risk of serious coronary heart disease (Cleland, 2002; Ray et al., 2002).

**References**


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