Cardioprotective effects of nitroparacetamol and paracetamol in acute phase of myocardial infarction in experimental rats

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Zhu, Yi Zhun, Chew Lan Chong, Shin Chet Chuah, Shan Hong Huang, Huey Shan Nai, How Teng Tong, Matt Whiteman, and Philip K. Moore. Cardioprotective effects of nitroparacetamol and paracetamol in acute phase of myocardial infarction in experimental rats. Am J Physiol Heart Circ Physiol 290: H517–H524, 2006. First published September 19, 2005; doi:10.1152/ajpheart.00572.2005.—We aimed to determine whether nitroparacetamol (NO-paracetamol) and paracetamol exhibit cardioprotective effects. Myocardial infarction (MI) was induced in rats, and drug treatment was started 1 wk before surgery. Mortality rate and infarct size at 2 days after MI were compared. Treatment groups included vehicle (saline), paracetamol (5 mg·kg−1·day−1) and NO-paracetamol (15 mg·kg−1·day−1). Mortality rates for vehicle (n = 80), paracetamol (n = 79), and NO-paracetamol (n = 76) groups were 37.5%, 21.5%, and 26.3%, respectively. Infarct size for the vehicle group was 44.8% (±6.1%) of the left ventricle (LV). For the paracetamol and NO-paracetamol groups, infarct size was 31.3% (±5.6%) and 30.7% (±8.1%) of the LV, respectively. Both paracetamol- and NO-paracetamol-treated groups showed increased activities of catalase and SOD compared with the vehicle group. They could attenuate endothelial, inducible, and neuronal nitric oxide synthase and cyclooxygenase-1 and -2 gene expression after MI. The observation indicates the potential clinical significance of the cardioprotective effects of these drugs.

infarct size; cardioprotection

ISCHEMIC HEART DISEASE (IHD) is a major cause of death and disability all over the world. If this trend is to be reduced or reversed, an improved understanding of the biological basis of IHD leading to the identification of new therapeutic interventions is essential. The possibility that NO donors may be of therapeutic benefit is also a recent conceptual advance. The idea that NO donors may produce biological effects quite different from classic NO donors is being investigated (19). The precise role of NO in many cardiovascular diseases remains unclear (1). Recently, an increasing number of studies (5, 20) have been conducted to evaluate the efficacy of a novel group of drugs—NO-releasing nonsteroidal anti-inflammatory drugs (NSAIDs). These studies were carried out in an attempt to search for new molecules that will retain the pharmacological profile (effective anti-inflammatory and analgesic actions) of NSAIDs but not their harmful effects (gastrointestinal dam-age). In the experiments conducted by Wallace (33), nitroaspirin was shown to be promising in the treatment of acute myocardial infarction (MI) with reduced side effects. It exerts similar anti-inflammatory and acute analgesic effects while sparing the gastrointestinal tract from injury. These studies suggested possible therapeutic benefits for nitroparacetamol (NO-paracetamol) similar to those of its parent drug (paracetamol). NSAIDs are one of the most largely prescribed classes of drugs and are also available over the counter. Paracetamol (acetaminophen) is well established as a leading nonprescription antipyretic analgesic drug. It was reported that the possible novel therapeutic applications for paracetamol include its use as an antioxidant to prevent atherosclerosis and cardiovascular disease by inhibiting the oxidation of low-density lipoproteins and to prevent the formation of cataracts (25). Although paracetamol shares a similar mechanism of action with conventional NSAIDs, its classification as a NSAID has been much debated. The controversy arises because, unlike NSAIDs, paracetamol displays no or very few anti-inflammatory properties. Recently, several groups reported that paracetamol could have protective effects after MI. It reduced infarct size in dogs (21) and in a rat ischemia-reperfusion model (8). However, whether NO-paracetamol would present effects similar to those of its parent drug in the acute phase of MI is still unknown. It is known that NO-paracetamol does not affect blood pressure (BP) or heart rate of anesthetized rats but has potency as an antipyretic agent similar to that of paracetamol (23). Thus we carried out this study to determine whether pretreatment with NO-paracetamol before acute MI would have beneficial effects like those of paracetamol on ischemic hearts. Evaluation of morphological changes and hemodynamic parameters as well as the possible biochemical and molecular mechanisms was carried out before and after drug (paracetamol and NO-paracetamol) treatment.

METHODS

Treatment procedure. Two hundred eighty-five male Wistar rats (200–250 g) were obtained from the Laboratory Animal Centre, National University of Singapore (NUS). Animals were housed under diurnal lighting conditions and fed standard rat chow and water ad libitum according to regulations of animal care and experiments by

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NUS, and the project was approved by the animal ethical committee. The rats were randomly assigned to three different treatment groups, paracetamol (5 mg·kg⁻¹·day⁻¹; n = 79), NO-paracetamol (15 mg·kg⁻¹·day⁻¹; n = 76), and vehicle (10% Tween 20 dissolved in 0.9% sodium chloride, 5 mg·kg⁻¹·day⁻¹; n = 80).

The rats were injected intraperitoneally once daily with their respective treatment drug. The treatment started 7 days before the surgery (day 8), and the tissue samples were collected 2 days after MI (day 10) for morphological and biochemical and molecular studies.

Animal model of myocardial infarction. MI was induced by a permanent ligation of the left coronary artery (29, 39). Treatment was continued for another 2 days after the surgery. At the end of the treatment period, hearts were collected, immediately immersed in liquid nitrogen, and stored at −80°C for further studies. Livers were used for antioxidant assays because those enzymes are mainly produced there. The antioxidant effects of paracetamol are considered as one of the protective factors in treating cardiac disease (25, 31, 40). Whether NO-paracetamol would have antioxidant effects in animals had not been reported previously.

Hemodynamic measurements. BP readings with the noninvasive blood pressure (NIBP) system (ML125/R, ADInstruments Powerlab System) and ECG readings (BioAmp amplifier, ADInstruments Powerlab System) were taken three times per rat throughout the animal model study (41). BP and ECG readings were recorded before the start of the experiment on day 1. ECG was measured for 1 min with an Animal BioAmp amplifier (ML 136, ADInstruments Powerlab System) as described previously (6). The rats were anesthetized with chloral hydrate (5 mg/kg ip) before ECG readings could be taken. Subsequently, readings were taken again on day 7 before the operation. The last pair of readings was measured before the death of the animal on day 10. Triplicate readings of BP and ECG were obtained for comparison as reported previously (6).

Infarct size. Infarct size was identified by 2,3,5-triphenyltetrazolium chloride ( TTC) staining as we previously reported (16, 30). In brief, the infarcted area was judged from both epicardial and endocardial sides, outlined on paper, cut, and weighed. The infarct size is defined as a ratio of the left ventricular (LV) infarct area to the whole LV area (16). The sizes of the LV and the infarct area were evaluated by computer with Scion Image (Scion), which is a well-recognized software used to analyze the size and intensity of irregular areas from tissue or gel (http://www.scioncorp.com).

Measurement of nitrite/nitrate concentration in plasma. Plasma (0.5 ml) was filtered with a Millipore Microcon and centrifuged at 10,000 rpm for 20 min at 4°C. Twenty-five microliters of 0.1 mM FAD, fifty microliters of 1 mM reduced NADP (NADPH), and ten microliters of nitrate reductase (10 U/ml) were added to the supernatant to convert the nitrate to nitrite and incubated at 37°C for 30 min. The reaction was terminated by 5 µl of lactate dehydrogenase and 50 µl of pyruvic acid, which oxidized the unreacted NADPH. The addition of Greiss reagents allowed the formation of a purple-pink azo dye, and the nitrite/nitrate (NOx) concentration was determined spectrophotometrically at 543 nm with Magellan software as reported previously (35).

Hepatic antioxidant assays. Hepatic antioxidant assays were carried out by testing for catalase (Cat), SOD, glutathione peroxidase (GPX), and glutathione S-transferase (GST) activities. Plasma was used for the measurement of NO, concentration.

By SOD, Cat, GPX, and GST activities were determined by an improved method as we reported recently (17). In brief, frozen liver tissue (1 g) was homogenized in 1 ml of phosphate buffer (10 mM, pH 7.5) with a Polytron homogenizer (Janke & Kunkel). SOD activity was determined based on the ability of the enzyme to inhibit autooxidation of pyrogallol (10 mM). The inhibition of pyrogallol oxidation by SOD was monitored at 420 nm in a spectrophotometer, and the amount of enzyme producing 50% inhibition was defined as one unit of enzyme activity. Cat was assayed by a mixture of 1 ml of 1:20 diluted supernatant A and 0.01 ml of ethanol after incubation in ice for 30 min. For the assay of GPX activity, a reaction mixture was made up with 100 µl of 1 M Tris·HCl EDTA buffer (5 mM EDTA, pH 8.0), 20 µl of glutathione (100 mM), 100 µl of glutathione reductase (10 U/ml), 100 µl of NADPH (2 mM), 10 µl of diluted supernatant B (1:20), and 660 µl of distilled H2O. After a 10-min reaction period at 25°C, 10 µl of i-butyl hydroperoxide was added to the mixture and mixed well. Immediately, the rate of disappearance of reduced NADPH was measured spectrophotometrically at 340 nm. GST was measured by a reaction mixture that was made up with 200 µl of phosphate buffer (pH 6.5), 20 µl of 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol (25 mM), and 680 µl of H2O. The activity of GST was estimated by measuring the change in optical density at 340 nm due to CDNB-glutathione, because CDNB-glutathione absorbs light at 340 nm. All assays were performed in triplicate at 25°C.

RNA extraction and RT-PCR amplification. Total RNA was extracted according to a standard protocol (39). One microgram of total RNA of each sample was reverse-transcribed into first-strand cDNA and amplified with a one-step RT-PCR kit (Qiagen) as reported by Mok et al. (22a). Briefly, 1 µg of RNA from each pooled sample was used in RT-PCR. The RT-PCR was carried out in a total volume of 20 µl containing 4 µl of Qiagen OneStep RT-PCR buffer, 0.8 µl of 2-deoxynucleotide 5'-triphosphate, 1.2 µl of sense primer, 1.2 µl of antisense primer, and 0.8 µl of Qiagen OneStep RT-PCR Enzyme Mix. RT-PCR was carried out in a thermocycler (GeneAmp PCR System 2700). First, the samples were incubated at 50°C for 30 min to allow reverse transcription for the synthesis of cDNA. Next, the samples were subjected to PCR amplification using primers specific for cyclooxygenase (COX)-1, COX-2, endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS), inducible NOS (iNOS), and GAPDH. Three-step PCR of denaturing, annealing, and extension was carried out at 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s, respectively.

The PCR products were in linear phase and had not yet reached plateau. The annealing temperature was set at 55°C for all six primers. The primer sequences and their product sizes are given in Table 1.

Statistics. Statistical comparisons between groups were given in Table 1. The primer sequences and their product sizes are given in Table 1.

RESULTS

Mortality. Mortality rates for vehicle (n = 80), paracetamol (n = 79), and NO-paracetamol (n = 76) groups were 37.5%, 21.5%, and 26.3%, respectively (Table 2). The paracetamol-treated group had significantly reduced mortality after MI (P < 0.05 compared with vehicle). It was observed that rats treated with NO-paracetamol had lower mortality rates compared with the vehicle group, but the P value is 0.135 compared with vehicle.

Hemodynamic parameters: BP and ECG. NO-paracetamol and paracetamol administered in this way did not have any discernible effects on BP or post-MI temperature in these animals. BP was measured at day 1 (the day treatment started; vehicle 122.7 ± 9.5, paracetamol 149.2 ± 26.3, and NO-paracetamol 128.9 ± 13.1 mmHg), day 7 (the day before surgery; vehicle 140.4 ± 14.4, paracetamol 143.2 ± 9.2, and NO-paracetamol 138.8 ± 6.1 mmHg), and day 10 (2 days after MI; vehicle 122.4 ± 31.6, paracetamol 127.9 ± 12.3, and NO-paracetamol 133.2 ± 11.5 mmHg) after the start of drug treatment. Thirty to thirty-three rats from each treatment group were randomly selected for the measurement of BP. It can be concluded that there was no significant change in BP throughout the treatment window.
ECG was measured on the first, seventh, and tenth days after the start of drug treatment. Six rats from each treatment group were randomly selected. There was no difference of ECG on days 1 and 7 (before MI) for each group (Fig. 1A). However, differences in ECG patterns were observed between the groups at day 10 (after MI; ECG charts are shown in Fig. 1, B–D). On day 10, all groups showed significant ST elevation, which is characteristic of MI. However, the ST elevation had the longest day 10, all groups showed significant ST elevation, which is characteristic of MI. However, the ST elevation had the longest day 10, all groups showed significant ST elevation, which is characteristic of MI. However, the ST elevation had the longest day 10, all groups showed significant ST elevation, which is characteristic of MI. However, the ST elevation had the longest day 10, all groups showed significant ST elevation, which is characteristic of MI. However, the ST elevation had the longest

Table 1. Gene sequences and corresponding product sizes of GAPDH COX-1, COX-2, eNOS, iNOS, and nNOS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product, bp</th>
<th>Accession No.</th>
</tr>
</thead>
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<tr>
<td>GAPDH</td>
<td>5′-CATGGTCTGATATGTTCCAGT-3′</td>
<td>349</td>
<td>XM_221353</td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-GCACTAAACAGTTGTGGTGTC-3′</td>
<td>350</td>
<td>S67721</td>
</tr>
<tr>
<td>COX-1</td>
<td>5′-CGAGGATGCTATCGAAGAG-3′</td>
<td>282</td>
<td>AF233596</td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-TCTGAGCTGTTGATGC-3′</td>
<td>423</td>
<td>NM_021838</td>
</tr>
<tr>
<td>COX-2</td>
<td>5′-CTGATADCCGGCTTGCTTG-3′</td>
<td>442</td>
<td>NM_012611</td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-ACTTGGCATGCTATG-3′</td>
<td>381</td>
<td>NM_052799</td>
</tr>
<tr>
<td>eNOS</td>
<td>5′-CTGGCAAGAGCCGATACACCA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-GCAATGTGACTCGAGAAAT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>5′-CTACCTCAGCTGGGAACACCTGG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-GGAGGCGGCTGATG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nNOS</td>
<td>5′-ATGGGAGACCCCCCTGAAGAC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′-TTCCAGGAGGGTCCACCACGG-3′</td>
<td></td>
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</table>

COX, cyclooxygenase; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS.

Table 2. Mortality rate and infarct size of vehicle-, paracetamol-, and NO-paracetamol-treated groups at end of 10-day treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Mortality, %</th>
<th>Infarct Size, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>37.5</td>
<td>44.8±6.1</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>21.5*</td>
<td>31.3±5.6*</td>
</tr>
<tr>
<td>NO-paracetamol</td>
<td>26.3</td>
<td>30.7±8.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *SE. NO-paracetamol, nitroparacetamol. *P < 0.05 compared with vehicle group.

Nitroparacetamol protects ischemic rat myocardium. Significant fewer capillaries were observed in the LVs of the vehicle group (246.1 ± 27.5%) compared with the paracetamol-treated (336.6 ± 17.9%) and NO-paracetamol-treated (302.9 ± 13.5%) groups (P < 0.05; Table 3). In the infarct area, myocardial necrosis was observed in the tissue sections obtained from all three groups (Fig. 3A). It was generally observed that there were actually fewer vessels in the vehicle group (Fig. 3B). Both paracetamol (Fig. 3C)- and NO-paracetamol (Fig. 3D)-treated groups had a comparable distribution of vessels in the noninfarct area of the LV.

Hepatic antioxidant assays. Antioxidant activities of Cat, GPX, GST, and SOD were measured in rat livers. Six liver samples from each treatment group were used in all four assays. In the Cat assay, a significant increase in enzyme activity was observed when both paracetamol (0.48 ± 0.02 U/mg protein)- and NO-paracetamol (0.61 ± 0.06 U/mg protein)-treated groups were compared with the vehicle group (0.32 ± 0.0 U/mg protein) (P < 0.05). In addition, there was also an increased enzyme activity when the NO-paracetamol-treated group was compared with the paracetamol-treated group (P < 0.05). A significant increase in SOD enzyme activity was observed when the NO-paracetamol-treated group (11.47 ± 1.81 U/mg protein) was compared with the vehicle group (8.27 ± 2.01 U/mg protein) (P < 0.05; Table 4). However, there was no significant change in enzyme activity among the three treatment groups in the GPX and GST assays except that there was a significant decrease in enzyme activity when the paracetamol group was compared with the vehicle group in the GST assay.

Gene expression of NOS and COX. NO-paracetamol had no effects on eNOS expression compared with vehicle-treated animals, whereas eNOS expression was significantly lower in the paracetamol group (0.86-fold) compared with the vehicle group (Fig. 4A).

In contrast, not all groups produced distinct iNOS and nNOS PCR product bands. The level of iNOS mRNA expression was detected in
the paracetamol group (0.23-fold) 48 h after MI, whereas expression in NO-paracetamol-treated rats was only 0.4-fold compared with the vehicle group (*P* < 0.05; Fig. 4C). A significantly lower level of nNOS production in both paracetamol- and NO-paracetamol-treated groups was observed (*P* < 0.05) compared with the vehicle group.

The paracetamol-treated group exhibited a significant reduction of COX-1 (0.75-fold) and COX-2 (0.79-fold) expression (*P* < 0.05) after MI compared with the vehicle group (Fig. 4, D and E). On the other hand, significant downregulation of COX-1 (0.84-fold) and COX-2 (0.85-fold) was also noted in the NO-paracetamol-treated group.

**DISCUSSION**

In the present study, NO-paracetamol and paracetamol were shown to display cardioprotective effects by lowering mortality rate and reducing infarct size and gene expression levels of NOS and COX. NO-paracetamol and paracetamol were seen to increase antioxidant enzyme activities and capillary density and to improve cardiac function (ECG) in the MI rats.

**Mortality and infarct size.** First, our present investigation reported that the mortality rates of NO-paracetamol- and paracetamol-treated groups were lower compared with the vehicle group. Paracetamol reduced mortality significantly compared with the vehicle group (*P* < 0.05). Although mortality of the NO-paracetamol group was not statistically significant, it showed a trend that NO-paracetamol could protect from ischemic heart damage after MI. NO-paracetamol and paracetamol also showed significantly reduced infarct size after MI (*P* < 0.05). Over the years, there has been a continuous addition of new cardioprotective agents to the list of agents that can reduce infarct size and lower mortality. Some of these compounds include angiotensin-converting enzyme inhibitors (38), angiotensin II type 1 receptor antagonists (39), antioxidants (16, 31, 40), apoptotic agents (34), and many others. The present study is the first report that NO-paracetamol and paracetamol could significantly reduce infarct size in rats after permanent MI. However, Merrill et al. (22) showed that paracetamol reduced infarct size in dogs and in rats after ischemia-reperfusion (8). Some of the theories put forward included the hypothesis that paracetamol diminishes hydrogen peroxide-induced ventricular dysfunction (21). Administration of NO-paracetamol before MI releases NO, which initiates a preconditioning-like phenomenon (28). This release of NO is hypothesized to be due to an esterase enzyme cleavage (23). NO would alleviate the consequences of MI, reducing infarct size and endothelial dysfunction (4). In the present study, we found that both NO-paracetamol and paracetamol could activate antioxidant enzymes and increase capillary density, which might also contribute to reduction of infarct size.

**Table 3. Capillary density**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Capillaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>246.1 ± 27.5</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>336.6 ± 17.9*</td>
</tr>
<tr>
<td>NO-paracetamol</td>
<td>302.9 ± 13.58*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3 rats/group). *P* < 0.05 compared with vehicle group.
of the slow release of NO, because the NOx concentration is increased in the NO-paracetamol group; this might be an effect paracetamol showed a significant attenuation of NOx level vivo in normotensive rats. NO-paracetamol has no apparent effects on vascular tone in with the above-mentioned studies, it can be established that been evaluated in hypertensive rats, and therefore the efficacy at high doses. On the other hand, the effects of both NO-paracetamol and paracetamol on BP and heart rate have not been evaluated in hypertensive rats, and therefore the efficacy of both drugs in the clinical setting in hypertensive patients remains to be investigated. From our current study together with the above-mentioned studies, it can be established that NO-paracetamol has no apparent effects on vascular tone in vivo in normotensive rats.

NOx concentration in plasma. Both paracetamol and NO-paracetamol showed a significant attenuation of NOx level compared with the vehicle group. The NOx level was not increased in the NO-paracetamol group; this might be an effect of the slow release of NO, because the NOx concentration is the stable end product of NO in plasma. After MI, cell necrosis leads to the release of oxygen radicals, proteases, and inflammatory mediators into the surrounding environment and triggers inflammation. Both paracetamol and NO-paracetamol sign-

ificantly reduce the NOx level in plasma, indicating that iNOS-derived NO is significantly lowered. Thus paracetamol and NO-paracetamol exhibit a cardioprotective effect by suppressing inflammation via inhibition of the COX pathway. Furthermore, the NOx level detected in the NO-paracetamol group was slightly lower than that in the paracetamol group, although the difference was not significant. This suggests that NO-paracetamol may have a slightly better anti-inflammatory effect than paracetamol, as NO has reactive oxygen species (ROS) scavenging properties. This suggestion can be supported by our present finding that the infarct size of the NO-paracetamol-treated group was lower than that of the paracetamol-treated group, and it seems that the exogenous NO acts by a mechanism that differs from iNOS-derived NO. However, the enzymatic activities of the NOS family should be examined to further determine which isoform elevation activities are responsible for the majority of NO production.

Capillary density. Under normal physiological conditions, capillary proliferation is generally atypical in the adult mammalian heart (7). However, this rare phenomenon may be observed under certain pathological conditions such as MI (16). Vascular endothelial cells proliferate during acute MI, resulting in angiogenesis that compensates for the limited blood flow to the ischemic myocardium. This repair mechanism corrects the imbalance between the perfusion capacity of coronary vessels and the need for oxygen and nutrients in the ischemic myocardium (37). It is still not clear why NO-paracetamol and paracetamol had an angiogenesis effect. It might be explained that NO could stimulate angiogenesis under the ischemic condition (24). This accounts for the significant increase in coronary blood vessels observed in the NO-paracetamol-treated group compared with both the paracetamol-treated and vehicle groups.

Antioxidant enzymes. It has been reported that the increased activities of antioxidant enzymes are beneficial after MI (16, 30, 31). In the present study, we observed that Cat activity in both NO-paracetamol- and paracetamol-treated groups and SOD activity in the NO-paracetamol-treated group were significantly higher than those in the vehicle group. However, GPX and GST activities were not increased. It can be seen that the main function of these antioxidant enzymes is to protect cells from ROS (40). Although these hepatic antioxidant enzymes are mainly in liver, they can be released into the systemic circulation and subsequently transported to various organs, including the heart, where they scavenge the free radicals present. As a result, therapeutic interventions that diminish free radical production or increase the antioxidant defense mechanism against free radicals may be implicated in the acute phase of MI treatment (40). The increased level of antioxidant enzymes stimulates the ability to scavenge free radicals.

Table 4. Antioxidant enzyme activities in liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Vehicle</th>
<th>Paracetamol</th>
<th>NO-Paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>0.32±0.04</td>
<td>0.48±0.02*</td>
<td>0.61±0.06††</td>
</tr>
<tr>
<td>GPX</td>
<td>0.62±0.10</td>
<td>0.50±0.12</td>
<td>0.74±0.28</td>
</tr>
<tr>
<td>GST</td>
<td>7.95±0.12</td>
<td>2.99±0.08</td>
<td>6.85±0.16</td>
</tr>
<tr>
<td>SOD</td>
<td>8.27±2.01</td>
<td>8.78±0.76</td>
<td>11.47±1.81*</td>
</tr>
</tbody>
</table>

Values (in U/mg protein) are means ± SE. Cat, catalase; GPX, glutathione peroxidase; GST, glutathione S-transferase. *P < 0.05 compared with vehicle group; †P < 0.05 compared with paracetamol group.
radicals, which may limit the myocardial damage inflicted by ROS, improving cardiac function (40).

**Gene expression of NOS and COX.** eNOS was found to be significantly downregulated in the paracetamol-treated group compared with the vehicle group. Felaco et al. (9) reported that eNOS gene expression was upregulated during hypoxia. eNOS-produced NO is believed to counteract the positive inotropic and chronotropic effect on β₁-adrenergic stimulation (11, 12). Downregulation of eNOS could therefore have positive inotropic effects on ischemic hearts.

![Gene expression chart](image)

Fig. 4. Gene expression of endothelial (eNOS; A), inducible (iNOS; B), and neuronal (nNOS; C) nitric oxide synthase, cyclooxygenase (COX)-1 (D), and COX-2 (E) in vehicle (inset, lane 1)-, paracetamol (lane 2)-, and NO-paracetamol (NO-para; lane 3)-treated groups after MI. Values are ratios with housekeeping gene GAPDH. *P < 0.05 compared with vehicle.
iNOS was weakly expressed in both paracetamol- and NO-paracetamol-treated groups. It has been reported that iNOS could cause cellular apoptosis and lead to contractile dysfunction (2, 15). In addition, mice lacking iNOS have improved contractile function and reduced cellular apoptosis after MI (27). In the present study, both paracetamol and NO-paracetamol significantly reduced iNOS expression level, suggesting downregulation of iNOS contributing to cardioprotective mechanisms after MI. This is also supported by other studies: biopsies from human hearts with dilated cardiomypathy and ischemic cardiomyopathy (13) demonstrate robust iNOS expression. The upregulation of iNOS in heart failure is thought to contribute to the pathophysiology of congestive heart failure, and iNOS upregulation is thought to be the result of the generation of several proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ (10). Downregulation of iNOS could therefore be cardioprotective after MI.

nNOS is a constitutively expressed gene in the orthosympathetic nerve terminals conjugating with the myocardium and atrioventricular node (3). In vagal nerve fibers, nNOS-derived NO mediates autonomic slowing of the heart (18). Takimoto et al. (32) showed that expression of nNOS in the atria was augmented and heart rate was parasympathetically decreased during acute MI in rats. In the present study, nNOS expression was significantly downregulated in both paracetamol- and NO-paracetamol-treated groups, which indicated that the decrease of nNOS mRNA level might allow the positive inotropic and chronotropic response to compensate for its reduced cardiac output and blood pressure at the early phase of cardiac remodeling.

COX-1 is expressed constitutively in most tissues and is involved in maintaining physiological functions, whereas COX-2, which is almost undetectable in basal conditions, is dramatically upregulated by proinflammatory and mitogenic stimuli, such as cytokines, growth factors, and bacterial toxins, thus playing a role in inflammation, infection, and malignant cell proliferation (36). Because inflammation constitutes an important feature of ischemic heart failure, especially in the initial phase of MI, inhibition of proinflammatory prostanooids through the use of a selective COX-2 inhibitor could significantly ameliorate myocardial injury and hence improve myocardial function. Therefore, in the present study, both paracetamol and NO-paracetamol exhibited a significant reduction of COX-2 expression in the MI heart compared with vehicle ($P < 0.05$), which shows that these two drugs could be potent inhibitors of COX-2. This is further supported by the studies of Saito et al. (26), which showed that COX-2 plays an important role in the pathogenesis of chronic heart failure secondary to MI, and suggests that therapy aimed at inhibiting myocardial COX-2 may prove beneficial in altering the course of this debilitating disease.

In conclusion, the observation that both NO-paracetamol and paracetamol produce a lower mortality rate than vehicle indicates the potential clinical significance of the cardioprotective effects of these drugs. In addition, NO-paracetamol also elicited cardioprotective outcomes by increasing capillary density within the ischemic myocardium and regulating gene expression of COX (COX-1 and COX-2) and NOS (eNOS, iNOS, and nNOS).

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