12-Lipoxygenase in Opioid-Induced Delayed Cardioprotection

Gene Array, Mass Spectrometric, and Pharmacological Analyses


Abstract—12-Lipoxygenase (12-LO) has been shown to be a factor in acute ischemic preconditioning (IPC) in the isolated rat heart; however, no studies have been reported in delayed PC. We characterized the role of 12-LO in an intact rat model of delayed PC induced by a δ-opioid agonist SNC-121 (SNC). Rats were pretreated with SNC and allowed to recover for 24 hours. They were then treated with either baicalein or phenidone, 2 selective 12-LO inhibitors. In addition, SNC-pretreated rats had plasma samples isolated at different times after ischemia-reperfusion for liquid chromatographic–mass spectrometric analysis of the major metabolic product of 12-LO, 12-HETE. Similar studies were conducted with inhibitors. Gene array data showed a significant induction of 12-LO message (P<0.05) after opioid pretreatment. This induction in 12-LO mRNA was confirmed by real-time polymerase chain reaction, and 12-LO protein expression was enhanced by SNC pretreatment at 24 hours relative to vehicle treatment. Both baicalein and phenidone attenuated the protective effects of SNC pretreatment on infarct size (50±4% and 42±3% versus 29±2%, P<0.05, respectively). No significant differences were observed in 12-HETE concentrations between baseline control and SNC-treated rats. However, 12-HETE concentrations were increased significantly at both 15 minutes during ischemia and at 1 hour of reperfusion in the SNC-treated rats compared with controls. Baicalein and phenidone attenuated the increase in 12-HETE at 1 hour of reperfusion. These data suggest that SNC-121 appears to enhance message and subsequently the activity and expression of 12-LO protein during times of stress, resulting in delayed cardioprotection. (Circ Res. 2003;92:676-682.)

Key Words: ischemia ■ opioids ■ late phase ■ gene array ■ lipid metabolites

Protection from myocardial infarction via ischemic preconditioning (IPC) has been described to be a biphasic event. The early phase occurs immediately after the IPC stimulus and lasts 1 to 3 hours; the late phase of protection is seen 12 to 24 hours after the initial stimulus and lasts up to 72 hours. We have previously shown that nonpeptide δ-opioid agonists induce a potent delayed cardioprotective effect that appears to be triggered by a burst of reactive oxygen species (ROS) that cannot be attributed completely to opioid receptor stimulation. The downstream signaling events that lead to delayed cardioprotection via opioids in terms of potential protein mediators remain unknown. It has been shown in an isolated rat heart model that acute preconditioning as well as protein kinase C activation stimulated the activity of 12-lipoxygenase (12-LO) to generate 12-HETE in the myocardium and that this increase in 12-HETE was associated with cardioprotection, because 12-LO inhibitors attenuated the recovery of left ventricular developed pressure. In addition, it has also been shown in isolated rat hearts that IPC causes the elevation of arachidonic acid (AA) and that 12-LO inhibitors attenuate the protective effects of IPC in terms of infarct size reduction. It has also been suggested in isolated myocytes that under basal conditions, AA metabolism produces a variety of products derived from cyclooxygenase (COX) and LO that include prostaglandins and HETEs but that under metabolic inhibition, AA is released without subsequent product formation by COX or LO. Therefore, it was suggested that impairment of AA metabolism represents a deficiency in signaling events that may be protective in nature and dependent on COX and LO metabolites.

No studies have considered the role of opioids on 12-LO activity in the myocardium, and no studies have looked at the...
mediation of delayed protection by 12-LO in an intact in vivo model. Initially, we performed a high-throughput screen for potential genes induced by opioid agonist pretreatment via gene array. On the basis of gene array results, we further assessed the role of 12-LO in SNC-induced delayed cardioprotection. We hypothesized that opioid pretreatment produces an enhanced activity of 12-LO during times of stress, which then functions as a mediator of delayed protection through the generation of metabolites of AA, such as 12-HETE.

Materials and Methods

Study Groups and Experimental Protocols
Male Sprague-Dawley rats (250 to 300 g) were randomly divided into groups and subjected to pretreatment with SNC-121, a nonpeptide μ-opioid agonist, followed by a 12-hour recovery period for gene chip and real-time polymerase chain reaction (RT-PCR) studies and a 24-hour recovery period for infarct (Figure 1), protein, and analytical studies. For gene chip analysis, a total of 5 rats in each group (SNC and vehicle) had total mRNA extracted and pooled. This pooled sample was then run on the gene chips (n=2 chips per group). *P<0.05 vs control.

Isolation of mRNA for Gene Chip and RT-PCR Analysis
Rats were pretreated with either vehicle or SNC-121 (0.1 mg/kg) and allowed to recover for 24 hours. Hearts were excised and homogenized, and protein was prepared for Western analysis. Representative Western blots for (1) positive control, (2) vehicle treated, and (3) SNC treated. A 41±8% increase in 12-LO expression was found in SNC-treated compared with vehicle-treated animals (n=3 per group). *P<0.05 vs control.

Affymetrix DNA Microarray Analysis
Affymetrix Gene Chip technology was performed as previously described. (See online data supplement for detailed Materials and Methods.) Briefly, cDNA was synthesized from total RNA and annealed to T7-(dT)24 primer. Reverse transcription was performed with Superscript II reverse transcriptase. Second-strand cDNA synthesis was performed with DNA polymerase I with the appropriate reagents. Synthesis of biotin-labeled cRNA was performed by in vitro transcription using the MEGAscript T7 IVT Kit (Ambion, Inc). The cRNA was fragmented and hybridized to the GeneChip Murine U74A Array Set (Affymetrix) in the appropriate hybridization solution. After washing and staining, probe arrays were scanned at 3-μm resolution with the GeneChip System confocal scanner (Hewlett-Packard) controlled by GeneChip 3.1 software (Affymetrix). Significant changes in gene expression were determined at a value of P<0.05 for control versus opioid-treated animals.

Real-Time PCR
RT-PCR was performed with the Quantitect SYBR Green kit (Qiagen) following the manufacturer’s instructions. Reaction volume was 25 μL, and 50 ng cDNA (from 3 vehicle-treated and 3 SNC-121–treated rats) was used as the template. 12-LO forward/reverse primers were at a final concentration of 1 μmol/L each. PCR was performed with an ABI Prism 7700 Detection System at an annealing temperature of 58°C. PCR products were visualized with gel electrophoresis to confirm a single product of the correct size (~100 bp). Data were analyzed by use of the SDS v1.9 software package to determine cycle threshold values.

Western Blotting
Hearts were excised (n=3 for each treatment), homogenized in lysis buffer containing protease inhibitors, and centrifuged at 1000 rpm for 20 minutes (Sorvall model RC-5B centrifuge with an SM-24 rotor) to remove nuclei and debris. The supernatant was collected, and protein concentration was determined by the Pierce assay. Fifty micrograms of protein or 2.5 μg of 12-LO positive control (Cayman Chemical) was loaded onto 10% Tris-HCl gels and after electrophoresis (200 V, 1 hour) was transferred to a PVDF membrane (100 V, 1 hour). Membranes were blocked with 10% milk solution, followed by probing overnight with a polyclonal primary 12-LO antibody (gift from Campbell Laboratory), followed by secondary antibody application (1:2500 BioRad and electrochemiluminescence (Amersham). Protein was detected by x-ray film, and densitometry was performed by NIH image 1.62.

General Surgical Procedure and Determination of Infarct Size
General surgery and determination of infarct size was performed as previously described. Briefly, rats were anesthetized and vessels cannulated for drug delivery and blood pressure measurement. A tracheotomy was then performed, and rats were ventilated with an artificial ventilator (Harvard Apparatus). Blood gases were monitored periodically (AVL 995 pH/Blood Gas Analyzer). A left thoracotomy was performed, followed by a pericardiotomy. A ligature was passed below the left descending coronary artery to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. Occlusion was elicited by pulling on the snare and clamping the snare onto the epicardial surface. After 2 hours of reperfusion, the coronary artery was again occluded. The area at risk (AAR) was determined by negative staining. The normal area and AAR were separated and stained with 1% 2,3,5-triphenyltetrazolium chloride in 100 mmol/L phosphate buffer (pH 7.4). Tissues were fixed overnight in 10% formaldehyde, and the infarcted tissue was dissected from the AAR with a dissecting microscope (Cambridge Instruments).

Preparation of Plasma Samples by Solid-Phase Extraction for Mass Spectroscopy
Solid-phase extraction columns (Varian C18 Bond Elut SPE columns) were preconditioned. Internal standards (5 μL), ethanol (176 μL/mL of sample), and glacial acetic acid (20 μL/ml of sample) were added to each sample. The samples were then vortexed and sonicated 3
times, after which they were centrifuged at 1500 rpm for ~5 minutes. The supernatant was loaded onto the solid-phase extraction column. The columns were washed, and samples were eluted with 6 mL of ethyl acetate. The ethyl acetate layer was removed from the water layer at the bottom of the reaction tubes and was dried under a stream of nitrogen gas. Samples were redissolved in 20 µL of acetonitrile, transferred to an insert in the sample vial, and analyzed by liquid chromatography–mass spectrometry (LC-MS).

Liquid Chromatography–Electrospray Ionization–Mass Spectrometry

Samples were analyzed by using LC–electrospray ionization (ESI)–MS (Agilent 1100 LC/MSD, SL model). The samples were separated on a reverse-phase C18 column (Kromasil, 250×2 mm) using water/acetonitrile with 0.01% acetic acid as a mobile phase at a flow rate of 0.300 mL/min. The mobile phase started at 35% acetonitrile for 1 minute, increased linearly to 68% acetonitrile in 18 minutes, increased to 100% acetonitrile in 15 minutes, and held for 10 minutes. Drying gas flow was 12 L/min, drying gas temperature was 350°C, nebulizer pressure was 35 psi gauge, vaporizer temperature was 325°C, capillary voltage was 90 V. The detection was made in the negative mode. For quantitative measurements, the m/z = 319 and 327 ions were used for 12-HETE and [2 H 8 ]12-HETE, respectively. The standard curves were typically constructed over a range of 5 to 500 pg per injection. The concentrations of 12-HETE in the samples were calculated by comparing their ratios of peak areas (12-HETE and [2 H 8 ]12-HETE) with the standard curves.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Affymetrix Gene Chips

Five rats were pretreated with SNC-121 or vehicle and allowed to recover for 12 hours. After recovery, hearts were excised and total mRNA was isolated. The isolated mRNA was then stored for later analysis. An Affymetrix gene platform was used for high-throughput screening of potential genes induced by SNC-121 that may mediate delayed cardioprotection. Gene chips of vehicle and experimental groups were run in duplicate, and genes that were significantly induced or repressed in the opioid-treated rats compared with vehicle treatment are shown in Table 1. 12-LO appears to be one of a small list of potential genes induced in response to opioid treatment that may contribute to delayed cardioprotection.

RT-PCR and Western Blotting Analysis for 12-LO

RT-PCR analysis was performed on mRNA isolated from hearts pretreated with vehicle or SNC-121 for 12 hours (n=3) to quantitatively confirm the results of the gene chip analysis. SNC-121 pretreatment showed a 1.7-fold induction of 12-LO mRNA compared with vehicle (P=0.03; data in online data supplement). A single PCR product was confirmed by agarose gel electrophoresis.

In addition, immunoblotting with a 12-LO–specific antibody revealed that 12-LO protein levels were induced significantly at 24 hours after SNC-121 pretreatment compared with vehicle (Figure 1).

Hemodynamic Data

The hemodynamic data for SNC-121–mediated delayed cardioprotection with and without 12-LO inhibition are summarized in Table 2. No significant differences were observed except for heart rate at 15 minutes of occlusion and 2 hours of reperfusion in the SNC-pretreated group given phenidone acutely.

Effect of 12-LO Inhibitors on SNC-Induced Delayed Cardioprotection

Rats were pretreated with SNC and allowed to recover for 24 hours. After the recovery period, baicalein (3 mg/kg) or phenidone (10 mg/kg) was administered 20 minutes before index ischemia (Figure 2). No significant differences were observed in left ventricle weight and AAR expressed as a percentage of the left ventricle. Both baicalein and phenidone significantly attenuated the protective effects of SNC pretreatment with respect to infarct size (50±4% and 42±3% versus 29±2%, P<0.05, respectively); however, the effect of SNC pretreatment on infarct size with phenidone given acutely was still statistically decreased compared with the control group, suggesting only a partial blockade (Figure 3).

Effect of Ischemia on AA and 12-HETE Production in Control and SNC-Pretreated Rats

Rats were pretreated with SNC and allowed to recover. Plasma samples were then collected at different time points after ischemia and reperfusion. Similar samples were taken from control rats. The samples were analyzed for AA and HETEs by LC-ESI-MS (Figure 4). Increasing amounts of stress released more free AA in plasma, but SNC pretreatment caused a significant release of AA only after 15 minutes of ischemia compared with the control rats at the same time point. Although at 1 hour of reperfusion there was a large increase in free AA compared with baseline, no differences

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**TABLE 1. Significantly Induced and Repressed Genes of Interest From Affymetrix Platform**

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>Probe Set</th>
<th>Ratio of Induction or Repression</th>
<th>t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-LO</td>
<td>S69383_at</td>
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<td>0.04</td>
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<tr>
<td>Signal transducer and regulator of transcription 5a1 (STAT5a1)</td>
<td>U24175_at</td>
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<td>0.04</td>
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<td>Solute transporter 22, member 7</td>
<td>L27651_g_at</td>
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<td>0.02</td>
</tr>
<tr>
<td>Similar to MEK kinase 3</td>
<td>rc_AA925300_at</td>
<td>2.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Repressed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EST228188</td>
<td>rc_Af231500_at</td>
<td>0.49</td>
<td>0.04</td>
</tr>
<tr>
<td>EST189755</td>
<td>rc_AA800258_at</td>
<td>0.45</td>
<td>0.01</td>
</tr>
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</table>
were observed between the control and SNC-pretreated groups. However, there was a statistically significant increase in 12-HETE production in the SNC-pretreated rats over the respective controls at 15 minutes after ischemia and at 1 hour of reperfusion. These data suggest that opioids enhance the expression and/or activity of 12-LO. LC-ESI-MS analysis showed no significant increase in 20-HETE, 12-HETE, or 5-HETE before ischemia.

**Effect of 12-LO Inhibitors on AA and 12-HETE Production in SNC-Pretreated Rats**

Rats were pretreated with SNC and allowed to recover for 24 hours. After the recovery period, rats were given baicalein or phenidone 20 minutes before index ischemia. Plasma was isolated at 1 hour of reperfusion, and AA and HETEs were determined (Figure 5). Treatment with 12-LO inhibitors did not attenuate the release of AA after ischemia. Free AA concentrations in plasma of control and SNC-pretreated rats at 1 hour of reperfusion were indistinguishable from those observed in SNC-pretreated rats that were given 12-LO inhibitors acutely. However, both 12-LO inhibitors significantly attenuated the generation of 12-HETE that was associated with SNC pretreatment. The amount of 12-HETE in the inhibitor groups was identical to that in the control group.

**Discussion**

We demonstrated that the delayed protective effects of SNC-121 are sensitive to 12-LO inhibition. These data suggest that 12-LO may potentially be a downstream mediator of delayed cardioprotection. We further demonstrated that SNC enhances the expression of 12-LO mRNA and protein expression and activity. RT-PCR confirmed that the message for 12-LO increases at 12 hours after SNC pretreatment, as suggested by gene chip analysis. Protein expression was enhanced at 24 hours after SNC pretreatment, the time point at which the stress stimulus of ischemia was given. Thus, enhanced message led to enhanced protein expression by SNC. In addition, opioid pretreatment may modulate the activity of 12-LO. Ischemia released free AA into plasma, and only pretreatment with SNC significantly enhanced the conversion of AA to 12-HETE during the ischemic stress. This enhanced generation of 12-HETE resulting from SNC pretreatment was significantly attenuated by 12-LO inhibitor treatment before the ischemic stress. These inhibitors were shown to block the delayed protective effects of SNC in the whole animal in terms of infarct size reduction. Together, these data suggest that 12-LO activity and expression are enhanced by

![Figure 2. Protocol bars. Rats were subjected to delayed preconditioning with SNC-121 (0.1 mg/kg). After a 24-hour recovery period, rats were treated with 2 different 12-LO inhibitors, baicalein (3 mg/kg) or phenidone (10 mg/kg), 20 minutes before index ischemia. Controls consisted of rats treated with vehicle. All rats underwent 30 minutes of index ischemia followed by 2 hours of reperfusion before infarct size was determined. TTC indicates triphenyltetrazolium chloride; OCC, occlusion.](image)

![Figure 3. SNC-induced delayed cardioprotection after acute treatment with 12-LO inhibitors. Control rats underwent 30 minutes of ischemia followed by 2 hours of reperfusion. Experimental rats were pretreated with SNC-121 (0.1 mg/kg) and allowed to recover for 24 hours. Baicalein (3 mg/kg) and phenidone (10 mg/kg) were given 20 minutes before index ischemia. The graph represents infarct size (IS) expressed as a percentage of AAR weight (IS/AAR, %, mean±SEM). The protective effects of SNC were attenuated after administration of 2 distinct 12-LO inhibitors after the recovery period. *P<0.05 vs control. +P<0.05 vs SNC-treated. n=5 to 25 rats per group.](image)
opioids such as SNC-121 and that 12-HETE generated by 12-LO during times of stress may partially mediate the delayed protective effects of opioids. It is likely that tissue levels of 12-LO expression are different from plasma expression of the enzyme. Further studies are necessary to confirm this. It is likely that opioids may function to stabilize the protein or may induce a comediator that enhances the activity of 12-LO at times of stress to induce protective signals.

Lipases such phospholipase C and phospholipase A are found in the circulation and have a key role in releasing AA during times of stress. Our current observations and those of Revtyak et al. show that the release of AA during stress does not necessarily allow for the induction of protective signals. AA must be properly metabolized, and it is these metabolites that have signaling potential. It is possible that products of 12-LO metabolism of AA are utilized during stressful events to induce protective signals, because inhibition of 12-LO not only attenuated the protection but also the generation of 12-HETE. It has been shown in previous studies that exogenous administration of a proximal metabolite of 12(S)-HETE, 12(S)-HpETE, improved recovery of left ventricular developed pressure in isolated rat hearts and that ischemic preconditioning is impaired in a 12-LO–knockout mouse. Thus, it appears that there is a causal link between 12-LO, 12-HETE production, and infarct size reduction.

The link between opioids and 12-LO has been established previously in the central nervous system. Studies have shown that morphine inhibits neurotransmitter release presynaptically and that this effect is mediated by 12-LO metabolites. In addition, the effects of opioids were enhanced by specific inhibition of COX-1 and 5-LO. This suggests that by inhibition of other enzymes that metabolize AA, there is a shift to the 12-LO pathway that results in an increased production of 12-LO metabolites, and it is these metabolites that may partially mediate the cardioprotective effects of opioids. Genomic analysis of hypoxia/reoxygenation in the neonatal rat brain showed an
upregulation of 12-LO, and this was confirmed by RT-PCR. Our present data and the study in the brain implicate 12-LO as a common mediator of tissue protection and may represent a novel therapeutic target for ischemic stress–induced injury to vital organs.

Numerous studies have suggested that NSAIDs may potentiate the effects of opioids in pain relief. The mechanism by which this occurs is a shift in AA metabolism. If there is synergism between opioids and 12-LO products, then inhibition of competing pathways that metabolize AA would result in more 12-LO metabolites and potentiation of opioid effects. This potentiation is suggested to occur through the interaction of 12-LO products such as 12-HETE with K channels. It is possible that in our system, opioids are acting by not only increasing the expression of 12-LO protein but also stabilizing the activity of 12-LO during times of stress. It is also possible that by selectively enhancing the production of 12-LO metabolites, opioids in the myocardium use a mechanism similar to that observed in neurons to facilitate signals that promote cell survival. However, the possibility exists that the source of the 12-HETE generated in the circulation is not ischemic myocytes but possibly leukocytes, nonischemic myocytes stressed by increased functional demand, or neurons, because SNC is centrally acting. These possibilities offer likely future directions for investigation.

A recent study looked at the role of baicalein in attenuating oxidant stress. It was found that baicalein is protective if it is given before reperfusion because it has ROS-scavenging ability that is separate from its ability to inhibit 12-LO. We observed that baicalein administered before ischemia attenuated the protective effects of SNC pretreatment and that this was associated with inhibition of 12-HETE formation. With these data and the observation that baicalein had no effect on infarct size alone, it appears that in our system, baicalein is working as a 12-LO inhibitor and is having minimal effects on scavenging ROS. We also showed that another pharmacologically distinct 12-LO inhibitor has an effect similar to that of baicalein, which suggests that there is 12-LO specificity in our model. However, it must be kept in mind that pharmacological agents have nonspecific effects that are often in opposition to what is experimentally desired. Therefore, we have corroborated in vivo findings with other evidence, such as mRNA expression and protein expression and activity, which in our model all point to the involvement of 12-LO in delayed cardioprotection.

In addition to 12-LO, the signal transducer and regulator of transcription 5a1 (STAT5a1), solute transporter 22, member 7, and a gene similar to MEK kinase 3 had a statistically significant induction, and EST228188 and EST189755 had a statistically significant repression. It has been shown in mice that isoforms related to STAT5a1 are involved in the late phase of protection induced after a preconditioning stimulus as well as acute ischemic preconditioning. Although these studies did not implicate STAT5a1 in the delayed phase of protection, it is likely that varied stimuli may utilize different isoforms to mediate the same effect.

Cytochrome c and p38 mitogen-activated kinase were repressed in response to SNC and may be of interest. Much controversy surrounds the exact role of p38 MAPK in the ischemic myocardium. There is evidence that supports the role of p38 as both protective and damaging. Inhibition of p38 has been shown to reduce ischemic injury and not inhibit the protective effects of acute preconditioning. Studies have also shown that there is no correlation between protection resulting from ischemic preconditioning and p38 phosphorylation, suggesting no causal link. In contrast, studies from our laboratory and others have shown that ischemic preconditioning involves p38 in the signaling events in the acute setting. In terms of delayed protection, the same controversy exists. Some have suggested that the delayed phase of protection induced by metabolic preconditioning is independent of p38 activation, whereas others have suggested the opposite: that p38 is a mediator of delayed protection induced by such varied stimuli as heat stress, adenosine, and anisomycin. It is likely that varied stimuli utilize the p38 MAPK pathway in different ways.

SNC-121 may also be antiapoptotic, because opioids have been shown to be both proapoptotic and antiapoptotic in neural and cancer cells, respectively. SNC appears to cause a repression of cytochrome c. Cytochrome c release from the mitochondria is considered to be one of the initiating factors of apoptotic signaling events. Cytochrome c may be released by receptor or chemically mediated pathways, and inhibition of cytochrome c release is protective and attenuates apoptosis. In isolated cardiomyocytes, it was shown that signaling events associated with KATP channel activity were associated with protection from apoptosis, and this led to an inhibition of cytochrome c release from the mitochondria. It is possible that SNC-121 pretreatment places the mitochondria in a protected state that prevents the induction of proapoptotic signals. This may be a result of the pro-oxidant environment that nonpeptide δ-opioid agonists may generate, their interaction with the sarcolemmal KATP channel in the triggering phase, or a combination of the two.

As with previous published data that show that the delayed cardioprotective effects of BW373U86, a structural analogue of SNC-121, are not completely inhibited by opioid receptor antagonism, similar studies show that the cardioprotective effects of SNC are not sensitive to opioid receptor antagonism (our personal observations). In addition, as with BW373U86, free radical scavenging with 2-mercaptopropionyl glycine completely attenuated the protective effects of SNC-121 (our personal observations). Therefore, it would seem likely that although nonpeptide δ-opioid agonists produce some effects through opioid receptor activation (eg, analgesia and cardiac hemodynamic effects), it would appear that we are describing a novel action of opioids that is independent of opioid receptor stimulation but dependent on ROS generation in mediating delayed cardioprotection. The likely target of opioids in generating ROS is pure speculation but may include the mitochondria or membrane-bound enzymes, such as NADPH oxidase, that function to generate reactive species. Further investigations need to be undertaken to clarify novel targets of nonpeptide δ-opioid agonists.

In conclusion, it appears that there is an interaction between SNC pretreatment and 12-LO. The interaction seems to be related to the presence of increased enzyme expression and perhaps with an increased activity of the enzyme during ische-
nia and after reperfusion. These data suggest that products of 12-LO metabolism of AA are downstream mediators of opioid-induced delayed cardioprotection. However, with the additional data generated by use of gene chip technology, there are numerous other pathways and mediators that must be worked out to obtain a full assessment of exactly how opioids mediate the phenomenon of delayed cardioprotection.

Acknowledgments
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References
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Methods

**Affymetrix DNA Microarray Analysis**

Affymetrix Gene Chip technology was performed as previously described.[Lockhart, 1996 #361] cDNA was synthesized from total RNA using Superscript Choice kit (GibcoBRL, Rockville, MD) and T7 polymerase (Mega Script T7 kit; Ambion, Austin, TX). Total RNA (7.9 µg) was annealed to T7-(dt)₂₄ primer (100 pmol/µl) at 70°C for 10 min. Reverse transcription (RT) was performed at 42°C for 1 hr in a mixture with final concentrations of 1X for the First Strand Buffer, 10 mM dithiothreitol, 500 µM each dATP, dCTP, dGTP, and dTTP, and 20,000 U of Superscript II reverse transcriptase per mL. The reaction was terminated by placing the tube on ice. Second-strand synthesis was carried out in 150 µl, incorporating the entire 20-µl first-strand reaction mixture and a 130-µl second-strand reaction mixture containing final concentrations of 1X for the Second Strand Buffer, 250 µM each dATP, dCTP, dGTP, and dTTP, 1.2 mM dithiothreitol, 65 U of DNA ligase per ml, 250 U of DNA polymerase I per ml, and 13 U of RNase H per ml. The mixture was incubated at 16°C for 2 hours, whereupon 2 µl of T4 DNA polymerase at 5 U/µl were added and incubation at 16°C was prolonged for 5 min. To terminate the reaction, 10 µl of 0.5 M EDTA was added. Following purification, the cDNA was precipitated with 5 M ammonium acetate and absolute ethanol at -20°C for 20 min. The pellet was resuspended in 1.5 µl of RNase-free water. Synthesis of biotin-labeled cRNA was carried out by *in vitro* transcription using the MEGAscript T7 In Vitro Transcription Kit (Ambion, Inc., Austin, TX). The reaction was carried out at 37°C for 5 hours in a mixture with 7.5 mM ATP, 7.5 mM GTP, 5.6 mM UTP, 1.9 mM biotinilated UTP, 5.6 mM CTP, 1.9 mM biotinylated CTP, 1X T7-Transcription Buffer, and 1 x T7-Enzyme Mix (Enzo Diagnostics, Farmingdale, N.Y.). The amplified cRNA was purified with an affinity resin column (RNeasy, Qiagen, Valencia CA).
The cRNA was fragmented by incubation at 94°C for 35 min in the presence of 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate. The fragmented cRNA was hybridized to the GeneChip Murine U74A Array Set (Affymetrix, Santa Clara, CA). A 220-µl hybridization solution of 1 M NaCl, 10 mM Tris (pH 7.6), 0.005% Triton X-100, 50 pM control oligonucleotide B2 (Affymetrix), control cRNA (Bio B 150 pM., Bio C 500 pM., Bio D 2.5 nM., and Cre X 10 nM.) (American Type Tissue Collection, Manassas, VA, and Lofstrand Labs, Gaithersburg, MD), 0.1 mg of herring sperm DNA per ml, and 0.05 µg of the fragmented cRNA per µl was heated to 95°C, cooled to 40°C, and clarified by centrifugation before being applied to chip. Hybridization was at 45°C in a rotisserie hybridization oven at 60 rpm for 16 h. Subsequent washing and staining of the arrays was carried out using the GeneChip fluidics station protocol EukGE-WS2. Briefly, the GeneChip probe arrays were washed 10 times at 25°C with Non Stringent Wash Buffer (6 x SSPE, 0.01% Tween20, 0.005% antifoam). The second wash consisted of 4 cycles of 15 mixes per cycle with Stringent Wash Buffer (100 mM MES (2-N-Morpholinoethanesulfonic acid, Sigma, St.Lous, MO), 0.1 M NaCl, 0.01% Tween-20) at 50°C. The arrays were stained for 10 min in streptavidin-phycoerythrin (SAPE) solution 1 x MES solution, 0.005% antifoam, 10 µg/ml SAPE (Molecular Probes, Eugene, OR), 2 µg/µl acetylated BSA (Sigma, St.Lous, MO) at 25°C. The post stain wash consisted of 10 cycles at 25°C in the fluidics station. The probe arrays were treated for 10 minutes in antibody solution (1 x MES solution, 0.005% antifoam, 2 µg/µl acetylated BSA, 0.1 µg/µl normal goat IgG (Sigma, St.Louis, MO), 3 µg/µl goat-anti-streptavidin, biotinylated antibody (Vector Laboratories, Burlingame, CA)) at 25 °C. The final wash consisted of 15 cycles of 4 mixes per cycle at 30°C in Fluidics station. Following washing and staining, probe arrays were scanned twice at 3µm resolution using the GeneChip System confocal scanner (Hewlett-Packard, Santa Clara, CA), controlled by GeneChip 3.1 software (Affymetrix).
Quality Control of Samples

The quality of the RNA from each sample was checked on a 1% agarose gel. To act as a quality control for the array studies several prokaryotic genes (e.g. bioB, bioC, and bioD are genes of the biotin synthesis pathway from the bacteria E.coli, Cre is the recombinase gene from P1 bacteriophage) were chosen to serve as hybridization controls. In addition, expression levels of 3' to 5' for both β-actin and GAPDH were evaluated; the 3'/5' ratio should be less than 3, according to the manufacturer’s instructions. All samples subjected to study met these several quality control criteria.

Data Analysis

Data analysis was performed using Affymetrix GeneChip 3.1 software. All genes not present in at least one of the groups at an expression level of at least 100 were removed from analysis. Significant changes in gene expression were determined at p<0.05 for control versus opioid-treated animals.

General Surgical Procedure

Rats were anesthetized using 120-150mg/kg Inactin intraperitoneally. The right jugular vein was cannulated for the delivery of saline. The right carotid artery was cannulated for the measurement of blood pressure and heart rate. Pressure and rate measurements were monitored using a Gould PE50 or PE23 pressure transducer connected to a Grass Model 7 polygraph. A tracheotomy was then preformed. The trachea was intubated with a cannula connected to a rodent artificial ventilator (model CIV-101, Columbus Instruments. Columbus, OH, or model 683, Harvard Apparatus, South Natick, MA). The rats were ventilated with room air at 38-45 breaths/min supplemented with O2. Atelectasis was prevented by maintaining a positive end-expiratory pressure of 5-10 mmH2O. Arterial pH, pCO2, and pO2 were monitored at control, 15 minutes of occlusion, and at 60 and 120 minutes after reperfusion using a blood gas system (AVL 995 pH/Blood Gas Analyzer).
Normal values were maintained by adjusting the respiratory rate and/or the tidal volume. Body temperature was maintained at 38°C using a heating pad.

Once heart rate and blood pressure stabilized, a left thoracotomy was performed at the fifth intercostal space. A pericardiotomy was then performed followed by adjustment of the left atrial appendage to locate the left coronary artery. A ligature (6-0 prolene) was passed below the left descending vein and coronary artery from the area immediately below the left atrial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. Occlusion for a period of 30 minutes was elicited by pulling on the snare and clamping the snare onto the epicardial surface using a hemostat. This resulted in left ventricular ischemia. Coronary artery occlusion was confirmed by epicardial cyanosis and a decrease in blood pressure. Reperfusion, for a period of 2 hours, was achieved by unclamping the hemostat and loosening the snare.

_Determination of Infarct Size_

After the 2-hour period of reperfusion, the coronary artery was again occluded using the snare. The area at risk (AAR) was determined by negative staining (Figure A-1). Patent blue dye was administered via the jugular vein to stain the non-occluded area of the left ventricle. The heart was excised, and the left ventricle was separated from the remaining tissue and cut into thin cross-sectional pieces. The normal areas were stained blue while the AAR remained pink. The normal area and AAR were separated and placed in different vials containing 1% 2,3,5-Triphenyltetrazolium chloride (TTC) in 100 mM phosphate buffer (pH 7.4). These vials were incubated at 37°C for 15 min. TTC is an indicator of viable and nonviable tissue. Tissues were fixed overnight in 10% formaldehyde, and the infarcted tissue was dissected from the AAR using a dissecting microscope (Cambridge Instruments). Infarct size (IS) and AAR were determined by gravimetric analysis. IS was expressed as a percentage of the AAR (IS/AAR).
Real-Time PCR Results

Real time-polymerase chain reaction (RT-PCR) analysis was performed on mRNA isolated from hearts pretreated with vehicle or SNC-121 for 12 hours (n=3) to quantitatively confirm the results of the gene chip analysis. SNC-121 pretreatment showed a 1.7-fold induction of 12-LO mRNA compared to vehicle (p=0.03). Rat # 1, 2, and 5 are SNC treated and #6, 7, and 8 are vehicle treated.

8th November 2002: SNC-121 and Vehicle rat cDNA real-time PCR analysis

- Rat 1, 2, 5 group is 1.7 fold more abundant than rat 6, 7, 8 group with a significance of P=0.03 Student's t test (n=3), i.e. a significant difference!
A single PCR product at the expected size of 100bp was confirmed by agarose gel electrophoresis for the primers used fore each rat sample. Specific lanes are 1=100kb ladder, 2-4=SNC treated rats, and 5-6=vehicle treated rats.

**Gene Chip Heat Image**

This image represents the data presented in Table 1 in image format. It represents in A. genes that were induced and in B. genes that were repressed in terms of mRNA expression.