Niacin promotes cardiac healing after myocardial infarction through activation of the myeloid prostaglandin D$_2$ receptor subtype 1

Deping Kong, Juanjuan Li, Yujun Shen, Guizhu Liu, Shengkai Zuo, Bo Tao, Yong Ji, Ankang Lu, Michael Lazarus, Richard M. Breyer, Ying Yu

Department of Pharmacology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin 300070, China (D.K., Y.S., Y.Y.); Key Laboratory of Food Safety Research, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China (D.K., G.L., S.Z., B.T., Y.Y.); Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China (J.L.); The Key Laboratory of Cardiovascular Disease and Molecular Intervention, Atherosclerosis Research Centre, Nanjing Medical University, Nanjing, Jiangsu 210029, China (Y.J.); Department of Cardiology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China (A.L.); International Institute for Integrative, Sleep Medicine (WPI-III-S), University of Tsukuba, Tsukuba City, Ibaraki 305–8575, Japan (M.L.); Department of Veterans Affairs, Tennessee Valley Health Authority, and Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA (R.M.B.).
Running title: Macrophage DP1 mediates resolution effect of niacin

Address correspondence to: Ying Yu, MD, PhD, Department of Pharmacology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, 300070, China; Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences, Shanghai, 294 Taiyuan Rd. Shanghai 200031, China; Tel. and Fax: 0086-21-54920970; E-mail: yuying@sibs.ac.cn; yuying@tmu.edu.cn

Number of text pages: 32

Number of tables: 3

Number of figures: 5

Number of references: 48

Number of words in the Abstract: 239

Number of words in the Introduction: 596

Number of words in the Discussion: 531

A recommended section assignment to guide the listing in the table of contents:
Cardiovascular; Drug Discovery and Translational Medicine; Inflammation, Immunopharmacology and Asthma
## Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg1</td>
<td>Arginase1</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethylcellulose</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DP1</td>
<td>Prostaglandin D&lt;sub&gt;1&lt;/sub&gt; receptor type 1</td>
</tr>
<tr>
<td>DP2</td>
<td>Prostaglandin D&lt;sub&gt;2&lt;/sub&gt; receptor type 2</td>
</tr>
<tr>
<td>Fizz1</td>
<td>Found in inflammatory zone 1</td>
</tr>
<tr>
<td>H-PGDS</td>
<td>Hematopoietic-type-PGDS</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-12β</td>
<td>Interleukin-12 beta</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>Lipocalin-type PGDS</td>
</tr>
<tr>
<td>Mac</td>
<td>Macrophage</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MRC1</td>
<td>CD206, mannose receptor, C type 1</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin D&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGDM</td>
<td>Prostaglandin D&lt;sub&gt;2&lt;/sub&gt; metabolite</td>
</tr>
<tr>
<td>PMNs</td>
<td>Peripheral monocytes</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YM1</td>
<td>Chitinase 3-like 3 (Chi3l3)</td>
</tr>
</tbody>
</table>
ABSTRACT

Niacin is a well-established drug used to lower cholesterol and prevent cardiovascular disease (CVD) events. However, niacin also causes cutaneous flushing side effects due to release of the proresolution mediator prostaglandin (PG) D2. Recent randomized clinical trials have demonstrated that addition of niacin with laropiprant (a PGD2 receptor subtype 1 [DP1] blocker) to statin-based therapies does not significantly decrease the risk of CVD events but increases the risk of serious adverse events. Here, we tested whether, and how, niacin beneficial effects in vivo myocardial ischemia requires the activation of the PGD2/DP1 axis. Myocardial infarction (MI) was reproduced by ligation of the left anterior descending branch of the coronary artery in mice. We found that niacin increased PGD2 release in macrophages and shifted macrophages to M2 polarization both in vitro and in vivo by activation of DP1 and accelerated inflammation resolution in zymosan-induced peritonitis in mice. Moreover, niacin treatment facilitated wound healing and improved cardiac function after MI through DP1-mediated M2 bias and timely resolution of inflammation in infarcted hearts. In addition, we found that niacin intake also stimulated M2 polarization of peripheral monocytes in humans. Collectively, niacin promoted cardiac functional recovery after ischemic myocardial infarction through DP1-mediated M2 polarization and timely resolution of inflammation in hearts. These results indicated that DP1 inhibition may attenuate the cardiovascular benefits of niacin.
INRODUCTION

Niacin, also known as vitamin B3 or nicotinic acid, is a safe, broad-spectrum lipid-lowering agent used to prevent cardiovascular events (Dunbar and Goel, 2016). Niacin inhibits free fatty acid (FFA) release from adipocytes (Carlson, 1963) and reduces plasma FFA levels in humans during fasting (Carlson and Oro, 1962) through its specific receptor GPR109A (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003). Niacin also suppresses hepatic low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and triglyceride (TG) synthesis through inhibition of diacylglycerol acyltransferase 2 (Ganji et al., 2004) and increases plasma high-density lipoprotein cholesterol (HDLc) independent of the GPR109A receptor or FFA suppression (Knouff et al., 2008; Zhang et al., 2012). In addition, niacin displays multiple anti-inflammation properties, including regulation of the expression of cell adhesion molecules (Tavintharan et al., 2009), nuclear receptors and scavenger receptors (Rubic et al., 2004; Knowles et al., 2006), through mechanisms that are unrelated to its effects on HDLc (Lukasova et al., 2011). Moreover, niacin suppresses atherogenesis in hyperlipidemic mice through GPR109A-mediated suppression of immune cells (Lukasova et al., 2011).

However, one major limitation of niacin is its induction of dermal side effects, e.g., facial flushing (Dunbar and Gelfand, 2010), resulting from stimulation of the GPR109A receptor in skin immune cells (Benyo et al., 2005). Vasodilatory prostaglandin (PG) D₂, perhaps PGE₂ as well, secreted in inflammatory cells, such as Langerhans cells, is thought to be involved in niacin-induced flushing through PGD₂ receptor subtype 1 (DP1) (Hanson et al., 2010). PGD₂ is an arachidonic acid metabolite derived from sequential reaction
of cyclooxygenases (COXs) and PGD$_2$ synthases (PGDSs) (Ricciotti and FitzGerald, 2011). Two distinct PGD$_2$ synthases (PGDSs), the haematopoetic PGDS (H-PGDS) and the lipocalin PGDS (L-PGDS), mediate the last regulatory steps in the biosynthetic pathway of PGD$_2$ production (Urade and Eguchi, 2002; Aritake et al., 2006). PGD$_2$ interacts mainly with two G-protein-coupled receptors: the DP1 receptor stimulates adenylyl cyclase through $G_\alpha_s$, whereas DP2 [initially called chemoattractant receptor T helper type 2 (CRTH2)] inhibits adenylyl cyclase through $G_\alpha_i$ and increases intracellular Ca$^{2+}$ (Malki et al., 2005; Spik et al., 2005). DP1 and DP2 are linked to different signaling pathways and appear to have distinct functions within the immune system (Kostenis and Ulven, 2006). DP1 antagonism suppresses niacin-induced vasodilation in both mice and humans (Cheng et al., 2006). Many studies have shown that cyclooxygenase-2-derived PGD$_2$ promotes resolution of inflammation (Rajakariar et al., 2007). DP1 is highly expressed in monocytes and macrophages (Rajakariar et al., 2007; Sandig et al., 2007), and PGD$_2$-induced macrophage M2 polarization facilitates inflammatory resolution through DP1-mediated suppression of Janus kinase 2 (JAK2)/signal transducer and activator of transcription (STAT) 1 signaling (Kong et al., 2016). However, whether niacin shapes macrophage polarization and accelerates inflammatory resolution through PGD$_2$ release remains unclear.

In the recent Heart Protection Study 2-Treatment of HDLc to Reduce the Incidence of Vascular Events (HPS2-THRIVE) trial, niacin therapy, combined with laropiprant (a DP1 antagonist), did not markedly decrease the risk of major cardiovascular events, but did increase the risk of serious side effects (Group et al., 2014). However, it is unclear whether the cardiovascular benefits of niacin can be confounded by DP1 inhibition (Song and FitzGerald, 2013; Dunbar and Goel, 2016). In this study, we evaluated the effects of
niacin on PGD₃ secretion and monocyte/macrophage M2 polarization through DP1 activation in mice using zymosan-induced peritonitis and myocardial infarction mouse models and analysis of the effects of niacin intake in humans. Our findings provide important insights into the effects of niacin on myocardial recovery from ischemic injury through activation of the DP1 receptor, suggesting that DP1 blockers may undermine the cardioprotective effects of niacin.

MATERIALS AND METHODS

Animals

DP1/F/F mice were maintained on a C57BL/6 genetic background and crossed with C57BL/6 LysMCre mice to generate DP1/F/FLysCre mice; these mice are referred to as Mac-DP1 KO mice (Kong et al., 2016). DP1/F/F littermates (hereafter referred to as WT mice) were used as experimental controls. All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, University of Chinese Academy of Sciences.

Reagents

IFNγ and IL-4 were purchased from Peprotech (Rocky Hill, NJ, USA). LPS, niacin, zymosan and thioglycolate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Peritoneal macrophage isolation and treatment

Peritoneal macrophages were induced and prepared by an intraperitoneal (i.p.) injection of 3% Brewer’s thioglycolate as described previously (Yang et al., 2010). Macrophages were allowed to adhere overnight (37°C, 5% CO₂) and washed with fresh medium to remove unattached cells before use. Macrophage polarization was subsequently induced with LPS.
(1 μg/mL) plus IFNγ (20 ng/mL) or IL-4 (20 ng/mL) and analyzed by reverse transcription polymerase chain reaction (RT-PCR) or western blotting, as described below. All primers used for RT-PCR are described in Tables 2 and 3.

**MI mouse model**

Left anterior descending (LAD) artery ligation was used in mice to induce myocardial infarction. Briefly, both male and female (6–8 weeks of age) mice were anesthetized with isoflurane (2%) using an induction chamber, and the LAD coronary artery was completely ligated to induce left ventricular ischemia (Gao et al., 2010).

**Cell sorting from post-MI hearts**

Mice were anesthetized and intracardially perfused with 40 mL ice-cold phosphate-buffered saline (PBS) to exclude blood cells. The heart was dissected, minced with fine scissors, and enzymatically digested with a cocktail of collagenase I (450 U/mL), collagenase XI (125 U/mL), DNase I (60 U/mL), and hyaluronidase (60 U/mL; Sigma-Aldrich) for 1.5 h at 37°C with gentle agitation. After digestion, the tissue was triturated and passed through a 70-μm cell strainer. Leukocyte-enriched fractions were isolated by 37–70% Percoll (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation, as described elsewhere (Yan et al., 2013). Cells were removed from the interface and washed with RPMI-1640 cell culture medium prior to staining with anti-CD45 and anti-CD11b antibodies and sorting into three populations: CD45⁺CD11b⁺ (myeloid cells), CD45⁺CD11blow (lymphocytes), and CD45⁺CD11blow (nonleukocytes). The CD45⁺CD11b⁺ population could be further divided into CD11b⁺F4/80⁺ macrophages and CD11b⁺Ly-6G⁺ neutrophils.

**Echocardiography**

Transthoracic echocardiography was performed at different time points after surgery using
an echocardiograph (Vevo2100). The investigator was blinded to group assignment. Mice were
anesthetized by isoflurane inhalation. Two-dimensional parasternal long axis views of the
left ventricle were obtained for guided M-mode measurements of the left ventricle (LV)
internal diameter at end diastole (LVDD) and end systole (LVDS), as well as the
interventricular septal wall thickness and posterior wall thickness.

**Flow cytometry**

Isolated peripheral blood cells and leukocytes isolated from the heart were analyzed by
flow cytometry. To block nonspecific binding of antibodies to Fcγ receptors, isolated
cells were first incubated with anti-CD16/32 antibody (BD Bioscience) at 4°C for 5 min.
Subsequently, the cells were stained with a mixture of the following antibodies at 4°C
for 30 min: anti-CD45-PE, anti-CD11b-FITC, anti-Ly6G-PE-Cy7, anti Ly6C-APC (eBioscience),
anti-F4/80-BV421, anti-CD206-APC (BioLegend). Human peripheral blood monocytes were
defined as SSClow CD14high cells. After staining with 7-amino-actinomycin D (Sigma) to
discriminate dead cells, flow cytometry was performed on a FACSCalibur (BD Bioscience)
and the data analyzed with FlowJo software (v.9).

**PGD₂ extraction and analysis**

Cell supernatants or peritoneal exudates (500 μL) were used for PG extraction after
protein quantification, as previously reported (Lu et al., 2015). An internal standard
(2 μL) was added to the sample in 40 μL of citric acid (1 M) and 5 μL of 10% butylated
hydroxytoluene, and the sample was then vigorously shaken with 1 mL solvent (normal
hexane:ethyl acetate, 1:1) for 1 min. The organic phase supernatant was collected after
centrifugation (6000 × g) for 10 min. The eluate was dried under nitrogen and analyzed
by electrospray triple/ quadruple LC-MS/MS (4000Q Trap AB Sciex). Chromatographic
separation was performed on an Agilent ZORBAX SB-Aq column (3.0×250mm, 5um) HPLC column, using 0.1% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B) as the mobile phase for binary gradient elution. The column flow rate was 0.4mL/min; the column temperature was 25°C, and the autosampler was kept at 4°C. The binary elution gradient was 30% B to 53% B in 15 minutes, and then to 90% B in 1 minute, maintained at 90% B for 3 minutes. The column was equilibrated for 10 min with the initial solvent composition between injections. PGD$_2$ was detected and quantified in negative ion mode, and the electrospray potential was maintained at -4.5 kV and heated to 500°C. For MS-MS analysis, PGD$_2$/internal standards were subjected to collision-induced fragmentation. PG production was normalized to total protein.

**PGDM Analysis**

Urinary prostanoid metabolite PGDM was extracted and quantitated as previously reported (Song et al., 2008). In brief, mouse urine was collected for 24 hours in metabolic cages with fasting treatment after niacin or CMC treatment from the third day post MI. Samples (100μl) were spiked with internal standard (10μl [²H$_6$]Tetranor PGDM) contained in acetonitrile. Two times of the urine volume of an aqueous solution of methoxyamine HCl (1 g/ml) was added and allowed to stand for 30 min at room temperature and then make capacity to 1ml by water. The solid phase extraction cartridge was conditioned with 1 ml of acetonitrile and equilibrated with 1 ml of water. The sample was applied to the cartridge, which was then washed with 1 ml of 5% acetonitrile in water and dried with vacuum for 15 min. The analyte and internal standard were eluted from the cartridge using 1 ml of 5% acetonitrile in ethylacetate. The eluate was collected and dried under a gentle stream of nitrogen. The resulting residue was then dissolved in 100 μl 10% ACN in water and passed
through small centrifugal filters with a 0.2-μm nylon membrane prior to analysis by mass spectrometry. The urinary creatinine was used to normalize the prostaglandin metabolites.

Collection of Human Plasma and Urine

Sixteen healthy volunteers (20–30 years old) without taking any NSAIDs within one week, were administered niacin (500 mg/day, orally) for three days. Peripheral blood samples were collected 12 hours before and after niacin intake. Urine samples were collected at the indicated time point [before (time 0) and after (time 1, 2, 3, 4, 6 and 12 h)] after niacin intake. Urinary PGD$_2$ metabolites (PGDM) were quantified by LC–MS/MS and peripheral monocytes sorted for gene expression analyses. The experiment was approved by the Human Ethics Committee of Shanghai Ruijin Hospital, and all the volunteers provided informed consent before the start of the experiment.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA samples from sorted cells or adherent macrophages were prepared using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) or TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed to cDNA with a Reverse Transcription Reagent kit (TAKARA, China), according to the manufacturer’s instructions. The resulting cDNA was amplified for 40 cycles. L32 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were amplified as internal controls. Each sample was analyzed in triplicate and normalized to a reference RNA. PCR products were confirmed by a single band of expected size on a 2% agarose gel. The primer sequences for PCR are summarized in Tables 2 and 3.

Western blotting
The protein concentrations of adherent or sorted macrophage lysates were determined using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal quantities of proteins were denatured and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels, transferred to nitrocellulose membranes, incubated with 5% skimmed milk for 1–1.5 h, and then incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted as follows: anti-phospho-STAT1 (58D6) (1:1000; Cell Signaling Technology), anti-STAT1 (1:1000; ABclonal Technology), anti-phospho-STAT6 (pY641) (1:1000; BD Biosciences), and anti-STAT6 (1:1000; ABclonal Technology). Anti-actin antibodies (1:2000; Sigma-Aldrich) were used as a loading control. The membranes were then incubated in horseradish peroxidase (HRP)-labeled secondary antibodies in blocking buffer for 2 h. Blots were developed using an enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA).

**Statistical Analysis**

All data are expressed as the means ± SEM. Data were analyzed in GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Two-tailed student’s t-testing and analysis of variance were used for comparisons between different groups. P-values < 0.05 were considered statistically significant.
RESULTS

Nichin promotes M2 macrophage polarization via activation of the PGD₂/DP1 axis

We previously found that PGD₂ triggers macrophage M2 polarization (Kong et al., 2016). To explore whether niacin alters macrophage polarization through PGD₂, we first tested PGD₂ generation in niacin-treated macrophages. As shown in Fig. 1A, niacin (3mM, 30 min) (Meyers et al., 2007) markedly enhanced PGD₂ secretion in both LPS/IFNγ and IL-4-challenged macrophages in vitro for induction of M1 and M2 polarization, respectively. Interestingly, niacin (3mM) effectively suppressed LPS/IFNγ-induced pro-inflammatory gene expression and enhanced IL-4-induced anti-inflammatory expression in wild-type (WT) macrophages (DP1⁺/⁺), but not in their DP1-deficient counterparts (DP1⁻/⁻; Mac-DP1 KO; Fig. 1, B and C). It has been proposed that STAT1 activation is indispensable for M1 macrophage polarization, whereas M2 macrophage differentiation requires STAT6 activity (Sica and Mantovani, 2012), also we have reported DP1 mediates M2 polarization through suppression of the STAT1 pathway and activation of the STAT6 pathway (Kong et al., 2016). Similarly, here we found that niacin pre-treatment depressed LPS/IFNγ-induced STAT1 activation (Fig. 1D) and enhanced IL-4-induced STAT6 activation (Fig. 1E) in macrophages in a dose-dependent manner, indicating niacin promotes macrophage polarization toward M2 status. Notably, these effects were disrupted by DP1 deletion (Fig. 1, F and G). Therefore, our findings indicated that niacin modulated macrophage polarization via activation of the PGD₂/DP1 axis.

Niacin accelerates resolution through DP1 in a zymosan-induced mouse model of peritonitis

Next, we examined the effect of niacin on zymosan-induced peritonitis in mice. Niacin (600mg/kg) (Wang et al., 1990; Nagai et al., 1994; Godin et al., 2012), was administered to mice 24 h after zymosan challenge (Fig. 2A), and peritoneal macrophages were isolated.
for gene expression analysis. Hematopoietic-type PGD synthase (H-PGDS) and DP1 expression levels were increased in peritoneal macrophages from zymosan-treated mice (Fig. 2B). Notably, niacin reduced both total infiltrated inflammation cells and macrophages at the later stages of zymosan-induced peritonitis in WT mice (Fig. 2, C and D), thereby accelerating resolution. Additionally, niacin markedly increased the CD11b+ F4/80+ CD206+ macrophage ratio (M2-like) at the resolution phase (both 48 and 72 h) in zymosan-treated mice (Fig. 2E). Moreover, consistent with our observations in vitro, niacin also suppressed pro-inflammatory gene expression [i.e., tumor necrosis factor-alpha (TNFα)] and induced anti-inflammatory gene expression [i.e., arginase 1 (Arg1), chitinase 3-like 3 (YM1) and CD206, mannose receptor, C type 1 (MRC1)] in peritoneal macrophages from WT mice (Fig. 2, F-I). None of these alterations were observed in Mac-DP1 KO mice (Fig. 2, C - G). Thus, niacin facilitated inflammatory resolution by promoting DP1-mediated macrophage polarization toward an M2-like state.

**Niacin improves post-MI recovery by promoting DP1-mediated inflammatory resolution**

We have confirmed that there was an increase of PGD2 in ischemic heart post MI (Fig. 3A). Again, niacin increased total body PGD2 production in mice, also upregulated both H-PGDS and DP1 expression in macrophages recruited to infarcted hearts at day 14 after experimental MI (Fig. 3, B and C). Interestingly, niacin alleviated the inflammatory response to ischemia and accelerated resolution by reducing the infiltration of CD45+ leukocytes and CD11b+ F4/80+ macrophages to the infarcted hearts in WT mice on days 7 and 14 after MI (Fig. 3, D and E, and Supplemental Fig. 1). Moreover, niacin also reduced the CD11b+ F4/80+ CD206+ macrophage ratio (M1-like) but increased the CD11b+ F4/80+ CD206+ macrophage ratio (M2-like) in WT mice on days 7 and 14 after MI (Fig. 3, F and G), suppressed
the expression of pro-inflammatory genes (i.e., IL-1β, TNFα, and nitric oxide synthase 2 [NOS2]), and increased the expression of anti-inflammatory genes (i.e., YM1, Arg1, and MRC1) in infiltrated macrophages from WT mice but not in Mac-DP1 KO mice (Fig. 3, H and I), indicating that niacin promoted M2 polarization in infarcted hearts through DP1. Accordingly, niacin dramatically restored cardiac function and tissue damage in WT mice but not in Mac-DP1 KO mice after MI (Fig. 3, J and K, Table 1 and Supplemental Fig. 2). Since DP1 activation may have gender differential responses in vasculature in mice (Song et al., 2012), we also compared the effect of Mac-DP1 deletion in cardiac recovery after MI between male and female mice. Again, Mac-DP1 deficiency markedly impaired cardiac recovery after MI in mice (Supplemental Table 1), but we failed to observe significant differences of heart functions between male and female Mac-DP1 KO mice before and after MI (Supplemental Table 1).

**Niacin modulates peripheral monocyte polarization in both mice and humans**

We also examined the effects of niacin (600 mg/kg) on polarization of peripheral monocytes in LPS-challenged mice (Fig. 4A). Expression of L-PGDS and DP2 were relatively low in mouse peripheral monocytes (Fig. 4, B–D). Interestingly, niacin enhanced H-PGDS and DP1 expression in peripheral blood monocytes (CD45+CD11b+Ly6G−Ly6C+; Fig. 4, B–D), but did not affect L-PGDS or DP2 expression (Fig. 4, B–D). As anticipated, LPS challenge induced pro-inflammatory M1 gene expression, which was blunted by niacin pretreatment in WT PMNs but not in Mac-DP1 KO PMNs (Fig. 4E). Moreover, niacin was only able to augment anti-inflammatory M2 gene expression in WT monocytes, while DP1-deficient cells were unaffected (Fig. 4F).
Next, to determine whether niacin had the same effects in humans, blood and urine samples were collected from healthy volunteers before and after a 3-day administration of niacin (Fig. 5A). As previously described (Song et al., 2008), niacin treatment resulted in substantial PGD$_2$ production (Fig. 5B). Interestingly, H–PGDS and lipocalin-type PGDS (L–PGDS) expression levels were significantly increased in human CD14$^+$ PMNs, but no significant effects were observed with respect to DP1 expression (Fig. 5, C and D). Accordingly, niacin induced the downregulation and upregulation of M1 and M2 markers, respectively, in human PMNs (Fig. 5, E and F), indicating that niacin also promoted human monocyte M2 polarization.

DISCUSSION

Niacin has been used in the treatment of dyslipidemia (Carlson and Oro, 1962; Carlson, 1963); however, adverse effects, including facial flushing, limit its use in part by enhancing PGD$_2$ synthesis (Dunbar and Gelfand, 2010; Hanson et al., 2010). Here, we found that niacin facilitated post-M1 healing through PGD$_2$ receptor DP1–induced M2 polarization and resolution of inflammation.

PGD$_2$ is involved in the resolution of inflammation (Gilroy et al., 1999). Consistent with this, H–PGDS deficiency results in impaired inflammatory resolution in mice (Rajakariar et al., 2007), and the pro-resolution effects of PGD$_2$ are believed to contribute to balancing the secretion of pro- versus anti-inflammatory cytokines through DP1 receptor (Rajakariar et al., 2007). We found that PGD$_2$ promotes M2 polarization and
resolution of acute inflammation, including MI, through DP1-mediated suppression of JAK2-STAT1 signaling (Kong et al., 2016). Interestingly, in vascular inflammation animal models, DP1 activation restrains aneurysm formation in males and atherogenesis in females (Song et al., 2012). We and others found DP1 mediates cardioprotection in both genders (Tokudome et al., 2009; Kong et al., 2016). Niacin stimulates PGD₂ biosynthesis in inflammatory cells through the GPR109A receptor (Benyo et al., 2005; Maciejewski-Lenoir et al., 2006). Accordingly, in this study, we showed that niacin promoted the resolution of inflammation and cardiac recovery from ischemia by activating DP1. In accordance with these observations, niacin inhibits various inflammatory reactions in different disease models (Godin et al., 2012).

Immediate-release niacin has been shown to ameliorate lipid profiles and improve outcomes in patients with acute myocardial infarction (Canner et al., 1986; Carlson and Rosenhamer, 1988). However, in two recent clinical trials (AIM-HIGH) (Investigators et al., 2011) and HPS2-THRIVE (Group et al., 2014), an extended-release (ER) alternative failed to recapitulate the benefits of the established cardioprotective regimen of niacin, despite significant increases in HDLc and reduction of TG levels in patients. These contradictory findings may be related to clinical design and outcomes, such as unbalanced use of statins and exetimibe (Song and FitzGerald, 2013) and possibly the dosage of the ER niacin alternative (Dunbar and Goel, 2016). In the HPS2-THRIVE trial, combinational use of the DP1 antagonist laropiprant may also have been an important factor (Song and FitzGerald, 2013). In experimental animals, pharmacological inhibition or genetic deletion of DP1 augments abdominal aneurysm formation, enhances angiotensin II-induced
hypertensive responses, and increases high-fat diet-induced atherosclerosis (Song et al., 2012; Strack et al., 2013). Moreover, the PGD2-DP1 axis mediates the cardioprotective effects of glucocorticoids against ischemia-reperfusion injury (Tokudome et al., 2009; Katsumata et al., 2014), and we found that DP1 expression in macrophages facilitates cardiac healing after MI by accelerating resolution (Kong et al., 2016). Thus, it is possible that DP1 inhibition by laropiprant could block the beneficial effects of niacin in mice with myocardial infarction. Indeed, in the HPS2-THRIVE trial, laropiprant was found to contribute to some serious adverse events, such as excessive bleeding and infection, given that laropiprant has high affinity for the thromboxane A2 receptor (Dallob et al., 2011) and that PGD2 has multiple anti-inflammatory properties.

In summary, our findings showed that niacin promoted cardiac recovery after MI through DP1-mediated M2 polarization and timely resolution of inflammation, suggesting activation of DP1 receptor may represent novel strategy for management of cardiovascular disease.

ACKNOWLEDGMENTS

We thank Maohua Shi, Kai Cao for the technical assistance.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Kong, Li, Shen and Yu.

Conducted experiments: Kong, Li, Shen, Zuo, Liu and Tao.

Contributed new reagents or analytic tools: Tao, Ji, Lu, Lazarus and Breyer.

Performed data analysis: Kong, Li and Shen.

Wrote or contributed to the writing of the manuscript: Kong, Shen and Yu.
REFERENCES


FOOTNOTES

This work was supported by the National Natural Science Foundation of China (81525004, 91439204, 81030004, 31200860 and 91639302), Shanghai Committee of Science and Technology Key Program (14JC1407400, 15140902000), Science and Technology Service Network Initiative (KFJ-EW-STS-099), the Postdoctoral Fellowship Program of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (2012KIP514 and 2013KIP312), and the National Institutes of Health (GM15431 and DK37097), as well as a Merit Award from the Department of Veterans Affairs, Vanderbilt University (1BX000616). Yong Ji and Ying Yu are Fellows at the Jiangsu Collaborative Innovation Center for Cardiovascular Disease Translational Medicine.

Address of person to receive reprint requests: Ying Yu, MD, PhD, Department of Pharmacology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, 300070, China; Tel. and Fax: 0086-22-83336627; E-mail: yuying@tmu.edu.cn.
Figure Legends

Figure 1. Niacin stimulates M2 macrophage polarization in vitro.

(A) Cultured primary peritoneal mouse macrophages were treated with LPS/IFNγ or IL-4 for 24 h after washing, then the cells were exposed to niacin (3mM) for 30 minutes, the supernatants were collected, and PGD₂ was analyzed by mass spectrometry. *p < 0.05, **p < 0.01 versus vehicle; n = 6. (B–C) Effects of niacin pretreatment (3mM, 30min) on pro-inflammatory (B) and anti-inflammatory (C) gene expression. (D–E) Effects of niacin pretreatment (0.5–3mM, 30min) on phosphorylation of STAT1 (D) and STAT6 (E) in peritoneal macrophages. (F–G) Effects of niacin pretreatment (3mM, 30min) on phosphorylation of STAT1 (F) and STAT6 (G) in WT and DP1-deficient peritoneal macrophages. *p < 0.05 versus vehicle; #p < 0.05 versus WT; n = 6. Data are expressed as mean ± SEM. All western blots were repeated three times, and other results were verified in two independent experiments. Statistical significance was determined using unpaired Student’s t tests.

Figure 2. Niacin accelerates resolution in zymosan-induced peritonitis through DP1 activation.

(A–I) Effects of niacin on zymosan-induced peritonitis in mice. Niacin (600 mg/kg, every 12 h) was administered 24 h after zymosan challenge (A), and peritoneal cells were harvested as indicated. PGDS and DP1 expression levels (B) were examined in macrophages, and the influence of niacin on total infiltrated inflammatory cells (C), total macrophages (D), M2 polarization ratio (E), and TNFα (F) and M2 (G–I) expression was assayed in macrophages isolated at the resolution stage of zymosan-induced peritonitis. CMC, carboxymethylcellulose. *p < 0.05, **p < 0.01 versus CMC (B) or as indicated (C–I); #p < 0.05 versus WT; n = 4–6. All data are expressed as mean ± SEM. P values were calculated
using two-way ANOVA followed by Bonferroni post-hoc tests (C–I) or unpaired Student’s t tests (B).

Figure 3. Niacin treatment improves recovery in post-MI mice by promoting DP1 activation-dependent M2 polarization and timely resolution.

(A) PGD$_2$ production in ischemic hearts after MI. Mice were underwent LAD ligation for 2 weeks, and then heart tissues were collected for PG extraction and examined by LC/MS/MS. **P < 0.01, n = 6. (B, C) Effects of niacin (600 mg/kg, twice daily) on urinary PGD$_2$ metabolites (PGDM) (B) and PGDS, DP1 expression in peripheral monocytes (C) in mice. *p < 0.05, **p < 0.01 versus CMC; n = 6. (D–G) Effects of niacin on inflammatory resolution and macrophage polarization in Mac-DP1 KO mice. Inflammatory cells were harvested from infarcted hearts, and total CD45$^+$ leukocytes (D), macrophages (E), and M1/M2 cell ratios (F, G) were analyzed. *p < 0.05, **p < 0.01 as indicated; n = 4–6. (H, I) Macrophages sorted at day 14 were used to examine pro-inflammatory (H) and anti-inflammatory (I) gene expression. *p < 0.05 versus CMC; #p < 0.05 versus WT; n = 6. (J) Representative echocardiography images with M-mode views of infarcted hearts after niacin treatment on day 14 post MI. Arrows and lines mark left ventricular inner diameters (LVID) in systole (dashed, white) and diastole (firm, blue). (K) Effects of niacin (600 mg/kg, twice daily) on heart functions post-MI in Mac-DP1 KO and WT mice. Niacin was administered from day 3 to 14 after MI. *p < 0.05; n = 8–10 per group. Data are presented as means ± SEM and are representative of two independent experiments. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc tests (D–G, K) or unpaired Student’s t tests (A, B, C, H, I).
Figure 4. Niacin stimulates peripheral monocyte M2 polarization via DP1 activation in LPS-challenged mice.

(A) Experimental design for niacin (600 mg/kg) treatment in LPS (8 mg/kg)–challenged mice. 

(B–F) Peripheral monocytes were sorted (B) to examine the mRNA levels of PGDS (C) and DP1 (D), pro-inflammatory (E) and anti-inflammatory (F) gene expression in the presence and absence of niacin. *p < 0.05 versus CMC, †p < 0.05 versus WT; n = 5–6. All graphs are shown as the mean ± SEM. Data are representative of two independent experiments. Statistical significance was determined using unpaired Student’s t tests.

Figure 5. Niacin intake promotes M2-like bias in human peripheral monocytes.

(A) Diagram of niacin intake and blood sample collection. (B) Urinary PGD2 metabolites (PGDM) were measured at different time points after niacin intake; n = 4. (C–F) CD14+ human peripheral monocytes (C) were monitored for PGDS, DP1 (D), pro-inflammatory (E), and anti-inflammatory (F) gene expression. *p < 0.05, **p < 0.01 versus before niacin; n = 15. All graphs are shown as the mean ± SEM. Statistical significance was determined using unpaired Student’s t tests.
# TABLES

Table 1. Effect of niacin treatment on recovery of Mac-DP1 KO mice 14 days post-MI.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Niacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=8)</td>
<td>Mac-DP1 KO (n=8)</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>35.74±1.992</td>
<td>18.32±2.144**</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>17.26±1.146</td>
<td>8.376±1.006**</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>4.710±0.1769</td>
<td>5.233±0.2329*</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>3.920±0.1516</td>
<td>4.798±0.2289**</td>
</tr>
<tr>
<td>BNP (pg/ml)</td>
<td>125.1±2.545</td>
<td>133.624±1.557*</td>
</tr>
<tr>
<td>CKMB (pg/ml)</td>
<td>19.82±0.211</td>
<td>21.581±0.231*</td>
</tr>
<tr>
<td>LVM/HW</td>
<td>0.691±0.016</td>
<td>0.593±0.152*</td>
</tr>
<tr>
<td>HW/BW</td>
<td>0.007±0.0003</td>
<td>0.009±0.0003**</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs WT; *p<0.05, **p<0.01 vs vehicle.

**EF:** Ejection Fraction; **FS:** Fractional Shortening; **LVIDd:** Left ventricular internal diameter at diastole; **LVIDs:** Left ventricular internal diameter at systole; **BNP:** Brain natriuretic peptide; **CK-MB:** Creatine kinase; **LVM:** left ventricular mass; **HW:** heart weight; **BW:** body weight; **All mice used are female; Data are presented as means ± SEM and are representative of two independent experiments. Statistical analysis was performed using a two-way ANOVA followed by a Bonferroni post-hoc test.**
Table 2. Primers for real-time PCR analysis in mice.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-PGDS</td>
<td>GGAAGAGCCGAAATTATTCGCT</td>
<td>ACCACTGCATCAGCTTGACAT</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>TGCAGCCAACCTTTCAACAAG</td>
<td>TGGTCTCACACTGTTTCTCTTCTCTTCT</td>
</tr>
<tr>
<td>DP1</td>
<td>AACCTCTATGACATGCACGGGGG</td>
<td>AAGGCTTGGAGGCTAGGTGCTCTCTCT</td>
</tr>
<tr>
<td>DP2</td>
<td>TCTCAACCAATCAGCACACCGA</td>
<td>GATGTAGCGGAGGCTAGAGGTGTCCTCT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGCTCTCCACCTCAATGGAC</td>
<td>GACAGGCTTGGCTGCTCTGTCTGCT</td>
</tr>
<tr>
<td>IL-12β</td>
<td>TGGTTGCGATCGTTTCTGCTCTTCT</td>
<td>ACAGGTCAGGCTACTGTTTCTCTCTCT</td>
</tr>
<tr>
<td>TNFα</td>
<td>ACGGCGATGGATCTCAAGAG</td>
<td>CGGACTCCGCAAAGTCTAAG</td>
</tr>
<tr>
<td>MCP1</td>
<td>TAAAAACCTGGATCGGAACCAA</td>
<td>GCATTAGCTCCAGATTTACGGGTT</td>
</tr>
<tr>
<td>NOS2</td>
<td>ACATCGACCCCTCCACAGAT</td>
<td>CAGAGGCTAGGCTTGCTCTCTCT</td>
</tr>
<tr>
<td>YM1</td>
<td>CAGGCTTGCCAATTTCTCTGAA</td>
<td>GTCTTGCTCATGTGTAAGGTA</td>
</tr>
<tr>
<td>MRC1</td>
<td>CTCTGTTCCAGCTATTGGACGC</td>
<td>CGGAATTCTGGGATTCAGGTTCTCTCT</td>
</tr>
<tr>
<td>IL10</td>
<td>TCAAGGATGACATCAAAGG</td>
<td>AGGCAAGGACTTCCCTCTCTCTCT</td>
</tr>
<tr>
<td>Dectin</td>
<td>GACTTCAGCAGCTACAGACCATCC</td>
<td>TTGTGCTGCAAATGCTAG</td>
</tr>
<tr>
<td>L32</td>
<td>TTAAGGCGAATGGCGGAAAC</td>
<td>TTGTGCTCCCTAACCAGATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCCTATTGACCTCAACTGCG</td>
<td>GAGGGCCATCCACAGTCTCTCTTG</td>
</tr>
</tbody>
</table>
Table 3. Primers for real-time PCR analysis in human samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>CGCCAGTGAATCCCTGTTGTT</td>
<td>AAGGTGGCATTGACAAACTCC</td>
</tr>
<tr>
<td>COX-2</td>
<td>CTGGCGCTCAGCCATACAG</td>
<td>CGCACTTATACTGTCATAATCCC</td>
</tr>
<tr>
<td>H-PGDS</td>
<td>ACCAGAGCCTAGCAATAGCAA</td>
<td>AGAGTGCCACAATAGCATCAAC</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>GGCGTTGTCCATGTGCAAG</td>
<td>GAATCTCCTGGTAGCTGAGAA</td>
</tr>
<tr>
<td>DP1</td>
<td>CTGGGCAAGTGCTCTCTAAG</td>
<td>CAACGAGTTGTCCAATGCAG</td>
</tr>
<tr>
<td>DP2</td>
<td>AAAAGGCTCGGGAAGGTTAAATG</td>
<td>AGGGGGAACCAAGAGAGAG</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>ATGATGGCTTTATTACAGTGCAA</td>
<td>GGTGGAGATTTCGTAGCTGGA</td>
</tr>
<tr>
<td>IL-12 β</td>
<td>ACCCTGACCACATCGAATCAA</td>
<td>TTGGCCTCGCATCTTAGAA</td>
</tr>
<tr>
<td>TNF α</td>
<td>CCTCTCTCTAATCAGCCCTCT</td>
<td>GAGGACCTGGGAAGTAGATGAG</td>
</tr>
<tr>
<td>NOS2</td>
<td>TTCAGTATCCAACACCTCAGAA</td>
<td>TGGACCTGGCAAGTTAAAATCCC</td>
</tr>
<tr>
<td>Arg1</td>
<td>GTGGAACTTGGCATCGGACAAC</td>
<td>AATCTGGCAACATCGGGAATC</td>
</tr>
<tr>
<td>MRC1</td>
<td>TCCGGGTGCTGTTCTCCTA</td>
<td>CCAGTCTGTTTTTGTGGGACT</td>
</tr>
<tr>
<td>Dectin</td>
<td>GGAAGCAACACATTGGGGAATGG</td>
<td>CTTGGTGGAGTGCACTGTC</td>
</tr>
<tr>
<td>Fizz1</td>
<td>CGTCTCCTGCTCCTCCTT</td>
<td>CTTTGACACTAGCACAAGAGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAGCGAGATCCCTCAAAAAT</td>
<td>GGCTTTGTGCTACTATTTCATGG</td>
</tr>
</tbody>
</table>
Figure 1

A

PGD$_2$

Vehicle LPS/IFN$\gamma$ IL-4

Level (ng/mg)

Vehicle Niacin

PGD$_2$

B

Vehicle Niacin LPS/IFN$\gamma$ IL-4

Relative expression to L32

Vehicle LPS/IFN$\gamma$ IL-4

* * *

* ** *

Level (ng/mg)

C

Vehicle Niacin

Vehicle LPS/IFN$\gamma$

Relative expression to L32

Vehicle Niacin

* * *

* ** *

Level (ng/mg)

D

Niacin LPS/IFN$\gamma$

p-STAT1

STAT1

$\beta$-actin

Niacin LPS/IFN$\gamma$

p-STAT1

STAT1

$\beta$-actin

0.5 1.5 3mM

E

Niacin IL-4

p-STAT6

STAT6

$\beta$-actin

Niacin IL-4

p-STAT6

STAT6

$\beta$-actin

0.5 1.5 3mM

F

WT Mac-DP1 KO

LPS/IFN$\gamma$

Niacin

p-STAT1

STAT1

$\beta$-actin

WT Mac-DP1 KO

p-STAT1

STAT1

$\beta$-actin

G

IL-4 Niacin

p-STAT6

STAT6

$\beta$-actin

IL-4 Niacin

p-STAT6

STAT6

$\beta$-actin
Figure 2

A

Time(h) 0 24 36 48 60 72
Zymosan (i.p.) Niacin (p.o.) Niacin (p.o.) Niacin (p.o.) Niacin (p.o.) Collect exudate

B

Relative expression to GAPDH (x10^{-2})

CMC Niacin

C

Total cell count

D

Macrophage

E

M2 Percentage

F

TNFα

G

Arg1

H

YM1

I

M2 Percentage

Relative expression to GAPDH

Cell count(x10^{6})

CD11b+F4/80^{+}(X10^6)

M2/M(\%)

Relative expression to GAPDH

Relative expression to GAPDH

Relative expression to GAPDH

Relative expression to GAPDH

Hours

Hours

Hours

Hours

Hours

Time(h) 0 24 48 72
Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate

Collect exudate
Figure 3.

A. PGD$_2$ levels in control and MI groups.

B. PGDM levels in CMC and Niacin groups.

C. Relative expression of H-PGDS, L-PGDS, DP1, and DP2.

D. CD45+ Leukocyte count changes over time with different treatment groups.

E. Macrophage count changes over time with different treatment groups.

F. M1% changes over time with different treatment groups.

G. M2% changes over time with different treatment groups.

H. Relative expression of IL1$\beta$, TNF$\alpha$, and NOS2 with different treatment groups.

I. Relative expression of YM1, Arg1, and MRC1 with different treatment groups.

J. LVEF changes over time with different treatment groups.

K. LVEF changes over time with different treatment groups.
Figure 4.

(A) Time (h) -2 0 12

Niacin (p.o.) LPS (i.p.)

Sort Peripheral Blood Monocytes

B

CD11b

CD45

Ly-6C

Ly-6G

C

D

E

F

Relative expression to GAPDH

H-PGDS L-PGDS

DP1 DP2

IL1β NOS2 MCP1

Relative expression to GAPDH

Relative expression to GAPDH

CMC Niacin

IL10 Fizz1 Dectin

Relative expression to GAPDH

Relative expression to GAPDH

CMC Niacin
Figure 5.

A. A timeline showing the administration of niacin on days 1, 2, 3, and 4, and the sorting of monocytes on days 1 and 4.

B. Graph showing PGDM levels over time (hours).

C. Flow cytometry plots showing the relative expression of markers to hGAPDH.

D. Bar graphs showing the relative expression of H-PGDS, L-PGDS, DP1, and DP2 before and after treatment.

E. Bar graphs showing the relative expression of TNFα, IL1β, IL12β, and NOS2 before and after treatment.

F. Bar graphs showing the relative expression of Arg1, Fizz1, MRC1, and Dectin before and after treatment.