Resolution of Inflammation by Resolvin D1 Is Essential for Peroxisome Proliferator-activated Receptor-γ-mediated Analgesia during Postincisional Pain Development in Type 2 Diabetes

Takayuki Saito, M.D., Maiko Hasegawa-Moriyama, M.D., Ph.D., Tae Kurimoto, B.S., Tomotsugu Yamada, M.D., Eichi Inada, M.D., Ph.D., Yuichi Kanmura, M.D., Ph.D.

ABSTRACT

Background: The wound healing process following acute inflammation after surgery is impaired in diabetes. Altered macrophage functions are linked to delayed tissue repair and pain development in diabetes. Although peroxisome proliferator–activated receptor (PPAR)-γ agonists are used to treat diabetes, their postoperative analgesic effects in diabetes have not been evaluated.

Methods: The PPARγ agonist rosiglitazone (rosi) was injected at the incision site of diabetic (db/db) mice with resolvin (Rv) D1, a lipid mediator involved in resolution of inflammation. Pain-related behavior, neutrophil infiltration, phagocytosis, and macrophage polarity were assessed for 7 days postoperatively.

Results: Rosiglitazone and RvD1 alleviated mechanical hyperalgesia in db/db (db) mice, whereas rosiglitazone alone did not alter mechanical thresholds on days 4 (db rosi + RvD1 vs. db rosi: 0.506 ± 0.106 vs. 0.068 ± 0.12) and 7 (0.529 ± 0.184 vs. 0.153 ± 0.183) after incision (n = 10 per group). In control m/m mice, the rosiglitazone-induced analgesic effects were reversed by knockdown with arachidonate 5-lipoxygenase small interfering RNA, but these were restored by addition of RvD1. In db/db mice treated with rosiglitazone and RvD1, local infiltration of neutrophils was markedly reduced, with an associated decrease in total TdT-mediated dUTP nick-end labeling cells. Acceleration of rosiglitazone-induced phenotype conversion of infiltrated macrophages from M1 to M2 was impaired in db/db mice, but it was effectively restored by RvD1 in db/db wounds.

Conclusions: In diabetes, exogenous administration of RvD1 is essential for PPARγ-mediated analgesia during development of postincisional pain. Resolution of inflammation accelerated by RvD1 might promote PPARγ-mediated macrophage polarization to the M2 phenotype. (ANESTHESIOLOGY 2015; 123:1420-34)

Type 2 diabetes is a major emerging epidemic strongly linked to cardiovascular disease and neuropathic disorders associated with sustained low-grade inflammation. Diabetic patients have increased susceptibility to infections after surgery, resulting in delayed wound healing.1 Sustained neuroimmune interactions associated with host defense prolong peripheral sensitization, leading to the development of pain hypersensitivity.2 Polymorphonuclear neutrophils (PMNs) followed by monocytes are the first leukocytes to accumulate at the site of inflammation. Monocytes can differentiate into macrophages that clear microbes, cellular debris, and apoptotic PMNs to resolve and repair and return the tissue to homeostasis. During resolution of inflammation, macrophages can acquire distinct functional phenotypes depending on the microenvironment of the inflamed site. Two well-established polarized phenotypes are referred to as classically activated (M1) and alternatively activated (M2) macrophages.3,4 M1 macrophages are recruited in the early phase, and they produce high levels of toxic intermediates.
associated with increased microbial activity and pronociceptive mediators such as inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α. In contrast, M2 macrophages expand after the initial M1 influx and have homeostatic functions linked to wound healing.

Peroxisome proliferator–activated receptor (PPAR)-γ is a member of the nuclear hormone receptor family and is implicated in mediating many metabolic, endocrine, and cardiovascular disorders. Activation of PPARγ potentiates polarization of circulating monocytes to M2 macrophages. PPARγ agonists are used clinically as antidiabetic agents, improving glucose and fatty acid metabolism by regulating innate immune responses, including macrophage polarization. We have previously reported that the PPARγ agonist rosiglitazone attenuates inflammatory pain after injection of complete Freund’s adjuvant and after surgery by increasing M2 macrophage recruitment. However, it is not clear whether the antidiabetic agent rosiglitazone has analgesic effects in type 2 diabetes.

Acute inflammation after injury is normally resolved in an organized series of events that ensure a return to homeostasis. Resolvins (Rvs) are known as critical lipid mediators for the resolution of inflammation by eliminating excess PMNs that have infiltrated into injured tissue. RvD1 is generated from docosahexaenoic acid, converted into 17(S)-hydroxy docosahexaenoic acid (17-HDHA) by arachidonate 15-lipoxygenase, and subsequently transformed by arachidonate 5-lipoxygenase (Alox5) into RvD1. 17-HDHA is a marker for the biosynthesis of RvD1 and promotes macrophage-mediated clearance of microbes and apoptotic cells. The level of 17-HDHA is low in wounds of diabetic (db/db) mice compared with wild-type m/m mice. Consistent with the hypothesis that impaired wound closure in diabetes is explained partly by disruption of the resolution process, including decreased levels of RvD1, it has been demonstrated that RvD1 decreases accumulation of apoptotic cells by enhancing macrophage phagocytosis in db/db mice.

In the current study, we investigated whether the antidiabetic PPARγ agonist rosiglitazone attenuated the development of postincisional pain through resolution of inflammation and phenotype shift of macrophages in diabetic mice.

**Materials and Methods**

**Animals**

Male BKS.Cg-Lepr^db/+Lepr^db/Jcl (db/db) mice aged 8 to 10 weeks were obtained from SLC Japan (Hamamatsu, Japan). The homozygous (db/db) mice were used as a model of type 2 diabetes, whereas Lepr+/Lepr^+ mice (m/m) served as nondiabetic controls. The Animal Research Committee of Kagoshima University approved all experimental procedures, which were implemented according to the guidelines of the National Institutes of Health and the International Association for the Study of Pain. Mice were housed in groups of four or five per cage with a 12-h light–dark cycle. Mice were randomly assigned to experimental conditions. The person performing the behavioral tests, immunohistochemistry, and quantitative polymerase chain reaction (PCR) was blinded to the type of treatment throughout the postoperative period but not to the genetic type of mice.

**Paw Incision Model**

The mouse hind paw plantar incision model was created as described previously. Mice were deeply anesthetized using 1.5 to 2.0% isoflurane (Abbott, Japan), which was inhaled by mice via a nose cone. A 5-mm longitudinal incision was made with a No. 11 blade through the skin and fascia of the plantar foot. The incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with a curved forceps leaving the muscle origin and insertion intact. The skin was apposed with a single mattress suture of 8-0 nylon. Rosiglitazone (Cayman Chemical, USA) was dissolved in a 1:3 solution of dimethyl sulfoxide (DMSO):phosphate-buffered saline (pH 7.2; 2.5 mg/ml) and then diluted in phosphate-buffered saline at a concentration of 1.25 mg/ml when rosiglitazone was injected alone (25 μg/20 μl). RvD1 (Cayman Chemical) was dissolved in 0.1% ethanol (2.0 μg/ml). Rosiglitazone (25 μg/10 μl), RvD1 (20 ng/10 μl), DMSO, 0.1% ethanol, and saline were injected at the incision site so that total injection volume was 20 μl. Rosiglitazone or DMSO was injected 30 min before the skin was incised. RvD1 or 0.1% ethanol was injected 15 min before rosiglitazone was injected. On postoperative days 1 and 2, rosiglitazone or DMSO was injected 3 h before behavioral tests were performed.

**siRNAs and Delivery**

Alox5 siRNA (Stealth RNAi; Invitrogen, USA) or its negative control small interfering RNA (siRNA) (4 μg in 1.0 μl) was prepared by mixing with the transfection reagent, in vivo-jet PEI (Polyplus-transfection, USA), dissolved in 9 μl of 5% glucose 10 min before injection. Intraplantar administration of siRNA (10 μl) was performed 3 h before incision. DMSO, rosiglitazone, or rosiglitazone and RvD1 was administered so that total injection volume was 10 μl at 30 min before incision. On days 1 and 2, siRNA was injected 3 h before behavioral tests were performed. The knockdown effects of Alox5 siRNA were examined by Western blotting (see fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B203). Samples taken from the hind paws of mice 3 h after injection of siRNA on day 2 were homogenized in radioimmunoprecipitation assay lysis buffer containing phosphatase inhibitor (Nacalai Tesque, Japan).
Protein samples (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were blocked with 2% milk and incubated overnight at 4°C with antibody to Alox5 (1:1,000; Abcam, USA) and β-actin (1:1,000; Cell Signaling Technology, USA). The blots were further incubated with horseradish peroxidase–conjugated secondary antibody (1:2,000; Cell Signaling Technology), developed with the Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA), and exposed to Hyperfilm (Amersham Bioscience, USA). The intensity of the selected bands was captured and analyzed by Image J 1.43u 2010 software (National Institutes of Health, USA). The optical density (OD) of Alox5 for each band was normalized to that of β-actin.

**Pain Behavior**

All behavioral experiments were performed by the same tester in a blinded manner. Withdrawal latencies to heat stimuli were assessed by applying a focused radiant heat source to an unrestrained mouse placed on a heat-tempered glass floor using the paw thermal stimulator (UCSD, USA). A thermal stimulus was applied to the plantar surface of each hind paw. Each mouse was tested at an interval of 2 to 3 min. The latency to thermal stimuli was calculated as the mean of three trials. A cutoff time was set at 20.5 s to avoid tissue damage. To evaluate tactile allodynia, calibrated von Frey filaments (0.08 to 2.0 g) were applied to the plantar surface of the hind paw from below the mesh floor. The 50% paw withdrawal threshold was determined using the up–down method (fig. 1A), as described previously.14

**Measurement of Paw Edema**

Postincisional edema, reflected by an increase in dorsal-to-ventral paw thickness, was measured using a microcaliper (Shinwa Measuring Tools, Japan). The mean of three measurements at each time point was used for analysis.

**Immunohistochemistry**

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and transcardially perfused with saline. Tissues were fixed in 4% paraformaldehyde overnight at 4°C and placed in 30% sucrose solution for 24 h at 4°C. Sections (30 μm thick) were incubated overnight with primary antibodies to neutrophil marker Gr-1 (1:100; AbD Serotec, United Kingdom), phagocytic macrophage marker CD68 (1:200; AbD Serotec), pan-macrophage marker F4/80 (1:100; Santa Cruz Biotechnology, USA), iNOS (1:500; Abcam), or CD206 (1:200; Santa Cruz Biotechnology) at 4°C overnight. Sections were incubated for 1 h at room temperature with secondary antibody labeled with Alexa Fluor 488 or 546 (1:500; Invitrogen, Life Technologies, USA) followed by nuclear staining with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, USA). Isotype controls to Gr-1, CD68 and F4/80 (rat immunoglobulin G [IgG]2b, 1:100; Santa Cruz Biotechnology), iNOS (polyclonal rabbit IgG, 1:500; Abcam), and CD206 (polyclonal goat IgG, 1:200; Santa Cruz Biotechnology) were included to check nonspecific background signal (see fig. 2, Supplemental Digital Content 1, http://links.lww.com/ALN/B203). TdT-mediated dUTP nick-end labeling (TUNEL) staining was performed on hind paw sections (30 μm thick) using the DeadEnd Fluorometric TUNEL System (Promega, USA) with coimmunostaining.

![Fig. 1](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/934691/)

**Fig. 1.** Local administration of rosiglitazone (rosi) ameliorated hypersensitivity to mechanical stimuli in m/m but not in db/db mice. (A) Schematic diagram of the plantar aspect of the mouse hind paw showing the site of behavioral experiments. (B) Withdrawal threshold to mechanical stimuli was measured for 7 days after incision. Two-way ANOVA (interaction factor: F_{12,180} = 16.47, P < 0.0001) followed by separate one-way ANOVA with Tukey post hoc test for comparing the mean withdrawal threshold at each time (before: F_{3,36} = 34.60, P < 0.0001; day 2: F_{3,36} = 0.4378, P = 0.7273; day 3: F_{3,36} = 2.063, P = 0.1223; day 4: F_{3,36} = 85.81, P < 0.0001; and day 7: F_{3,36} = 34.81, P < 0.0001). (C) Withdrawal latency to thermal stimuli. Two-way ANOVA (interaction factor: F_{12,180} = 0.1763, P = 0.9991). (D) Paw edema after incision. Two-way ANOVA (interaction factor: F_{12,180} = 0.6935, P = 0.7565). The results are presented as mean ± SD. **P < 0.001, ****P < 0.0001.
of CD68 (1:200) followed by labeling with Alexa Fluor 546 (1:500) and DAPI. Fluorescent images were obtained using a LSM700 imaging system (Carl Zeiss, Germany). The number of Gr−1+, CD68+TUNEL+, total F4/80+, F4/80+iNOS+, F4/80+CD206+, and iNOS−CD206− cells with clearly visible cell bodies stained with DAPI in the subcutaneous tissue was evaluated using Image J 1.43u 2010 software (National Institutes of Health).

Quantitative PCR
Total hind paw RNA was extracted using Sepazol reagent (Nacalai Tesque). The synthesis of first-strand complementary DNA (cDNA) was performed using High-Capacity RNA-to-cDNA kit (Applied Biosystems, Life Technologies). TaqMan assays were performed for quantification of Tnf (assay ID: Mm00443260_g1) and transforming growth factor (TGF)-β1 (Tgfβ1) (assay ID: Mm01178820_m1) using TaqMan Fast Advanced Master Mix (Applied Biosystems) on an ABI Prism StepOnePlus Real-time PCR System (Applied Biosystems). Target gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase.

Statistical Analysis
Sample size was determined based on our previous studies.7,8 Values are presented as the mean ± SD. For the evaluation of Alox5 knockdown by siRNA, unpaired Student t test was performed. Differences among multiple groups were analyzed by two-way ANOVA to test for the significance of changes in time and among the different treatment groups, followed by separate one-way ANOVA with Tukey post hoc test to compare the difference among pairs of treatments. Results in neutrophil infiltration were compared by one-way ANOVA with Tukey post hoc test. Statistical analyses were conducted using GraphPad Prism version 5.0 (USA). All probability values were two tailed, and a P value less than 0.05 was considered statistically significant.

Results
Rosiglitazone Ameliorates Postincisional Mechanical Hyperalgesia in Combination with RvD1 in Diabetic Mice
To evaluate the effects of rosiglitazone on the development of postincisional hyperalgesia in diabetes, rosiglitazone was injected locally into the hind paw 30 min before and on days 1 and 2 after plantar incision. It has been reported previously that db/db mice develop mechanical allodynia at 6 to 12 weeks of age,15 which is consistent with our finding that db/db mice exhibited a decreased mechanical threshold compared with vehicle in db/db mice (db rosi vs. db vehicle: 0.03 ± 0.025 vs. 0.017 ± 0.017). No differences were observed in thermal latency or paw edema between m/m and db/db mice regardless of the injections of rosiglitazone (fig. 1, C and D). In contrast to the effects of rosiglitazone alone on mechanical hyperalgesia, rosiglitazone markedly increased mechanical threshold in db/db mice when coadministered with RvD1 on days 4 (db rosi vs. db rosi + RvD1: 0.068 ± 0.120 vs. 0.506 ± 0.106, P < 0.0001) and 7 (db rosi vs. db rosi + RvD1: 0.153 ± 0.183 vs. 0.529 ± 0.184, P < 0.01) (fig. 2A). Coadministration of RvD1 with rosiglitazone did not alter withdrawal latency to heat stimuli (fig. 2B) or paw edema (fig. 2C). In this study, DMSO and 0.1% ethanol were used as solvents of rosiglitazone and RvD1, respectively. It is reported that DMSO injected 30 min before formalin injection enhances formalin-induced nociception,16 whereas 0.1% ethanol does not change the mechanical threshold.17 In contrast to the previous study, intraplantar injection of DMSO with 0.1% ethanol did not alter mechanical threshold (fig. 2A) or thermal latency (fig. 2B) but increased paw thickness in both m/m and db/db mice (fig. 2C), which was not restored by the administration of rosiglitazone (fig. 1D).

In addition, to examine whether RvD1 mediates PPARγ-induced analgesia, siRNA specific for Alox5, which is involved in the biosynthesis of lipid mediators including RvD1,18 was administered with rosiglitazone in m/m mice to block the conversion of 17-HDHA into RvD1 at the incision sites. We confirmed that the administration of Alox5 siRNA significantly decreased the expression levels of Alox5 compared with that of control siRNA (m/m control siRNA vs. m/m Alox5 siRNA: 1.391 ± 0.515 vs. 0.758 ± 0.185, P = 0.0177) (see fig. 1, Supplemental Digital Content 1, http://links.lww.com/ALN/B203). Coadministration of Alox5 siRNA with rosiglitazone significantly decreased the effects of rosiglitazone on the mechanical threshold in m/m mice on days 4 (m/m control siRNA + rosi vs. m/m Alox5 siRNA + rosi: 0.607 ± 0.398 vs. 0.007 ± 0.002, P < 0.01) and 7 (m/m control siRNA + rosi vs. m/m Alox5 siRNA + rosi: 0.816 ± 0.530 vs. 0.107 ± 0.173, P < 0.01) (fig. 3A). The decrease in the mechanical threshold was significantly recovered by coadministration of RvD1 with rosiglitazone under knockdown of Alox5 with siRNA to levels approximately equal to those in mice treated with control siRNA and rosiglitazone on days 4 and 7, suggesting that RvD1 is essential for PPARγ-mediated analgesia to mechanical stimulation. Coadministration of Alox5 siRNA with rosiglitazone did not alter withdrawal latency to heat stimuli (fig. 3B) or paw edema (fig. 3C). These results suggest that among various lipid mediators synthesized by Alox5, RvD1 might have a major role in PPARγ-mediated analgesic effects against incision-induced mechanical hyperalgesia.

Coadministration of RvD1 with Rosiglitazone Attenuates Infiltration of PMNs
Prolonged infiltration of neutrophils contributes to pain sensitization after surgical incision.19 We investigated the effects
of rosiglitazone on infiltration of PMNs into the subcutaneous tissue of incision wounds in db/db mice. PMNs identified immunohistochemically with the antibody to Gr-1 were rarely observed in the intact hind paws of m/m and db/db mice (fig. 4, A and B). Accumulation of Gr-1+ PMNs was markedly increased in db/db compared with m/m mice regardless of rosiglitazone injection (fig. 4B). In contrast to rosiglitazone-treated incision sites, infiltration of Gr-1+ PMNs was markedly reduced when RvD1 was exogenously coadministered with rosiglitazone in db/db mice on days 2 (db rosi vs. db rosi + RvD1: 1,243 ± 252 vs. 333 ± 214 cells/mm², P < 0.0001) and 7 (db rosi vs. db rosi + RvD1: 1,339 ± 64 vs. 273 ± 58 cells/mm², P < 0.0001), consistent with the previous report that RvD1 limits neutrophil infiltration.20 However, administration of RvD1 alone was not sufficient to inhibit PMN infiltration in db/db mice. These data suggest that exogenously administered RvD1 and PPARγ activation by rosiglitazone are critical for clearance of PMNs in db/db mice.

**Fig. 2.** Rosiglitazone (rosi) attenuated mechanical hyperalgesia in combination with resolvin D1 (RvD1) in db/db mice. (A) Withdrawal threshold to mechanical stimuli. Two-way ANOVA (interaction factor: F(36,390) = 7.95, P < 0.0001) followed by separate one-way ANOVA with Tukey post hoc test for comparing the mean withdrawal threshold at each time (before: F(9,78) = 10.60, P < 0.0001; day 2: F(9,78) = 1.647, P = 0.1165; day 3: F(9,78) = 2.978, P = 0.0042; day 4: F(9,78) = 13.77, P < 0.0001; and day 7: F(9,78) = 21.80, P < 0.0001). (B) Withdrawal latency to thermal stimuli. Two-way ANOVA (interaction factor: F(36,390) = 0.6565, P = 0.9383). (C) Paw edema after incision. Two-way ANOVA (interaction factor: F(36,390) = 2.377, P < 0.0001) followed by separate one-way ANOVA with Tukey post hoc test for comparing the mean paw thickness at each time (before: F(9,78) = 3.976, P = 0.0003; day 2: F(9,78) = 11.15, P < 0.0001; day 3: F(9,78) = 10.14, P < 0.0001; day 4: F(9,78) = 10.07, P < 0.0001; and day 7: F(9,78) = 7.618, P < 0.0001). The results are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. DMSO = dimethyl sulfoxide.
in accumulation of PMNs mediated by coadministration of rosiglitazone with RvD1 might contribute to the attenuation of pain development in the later phase of the postoperative period in db/db mice.

**Apoptotic Cells Engulfed by Macrophages at Incised Sites Are Decreased by Rosiglitazone in Combination with RvD1 in db/db Mice**

Clearance of apoptotic neutrophils by macrophages, a process termed efferocytosis, is an important step in preventing tissue necrosis and chronic inflammation. Therefore, we used the TUNEL assay to evaluate whether apoptotic cells were efficiently cleared by phagocytic macrophages that expressed CD68 from the wounds of db/db mice after coadministration of rosiglitazone and RvD1 (fig. 5A). The number of CD68+TUNEL+ cells was increased at rosiglitazone- and/or RvD1-treated sites compared with vehicle-treated sites of m/m mice (fig. 5B), consistent with the decrease in total TUNEL+ cells on day 2 (fig. 5C). In contrast, total TUNEL+ cells were decreased in db/db mice treated with rosiglitazone and RvD1, whereas rosiglitazone alone did not alter the number of total TUNEL+ cells on days 2 (db rosi + RvD1 vs. db rosi: 24 ± 27 vs. 129 ± 78 cells/mm², P < 0.01) and 7 (db rosi + RvD1 vs. db rosi: 23 ± 24 vs. 130 ± 66 cells/mm², P < 0.01). Although the total number of CD68+ cells did not differ between the groups (fig. 5D), CD68+TUNEL+ cells were increased in db/db mice by coadministration of RvD1 and rosiglitazone compared with rosiglitazone alone on day 2 (db rosi + RvD1 vs. db rosi: 37 ± 19 vs. 12 ± 10 cells/mm², P < 0.01) but not on day 7 (db rosi + RvD1 vs. db rosi: 33 ± 21 vs. 24 ± 19 cells/mm²). Total TUNEL+ cells were decreased in db/db mice treated with rosiglitazone and RvD1, implying that RvD1 and PPARγ might enhance the process of macrophage-mediated clearance of apoptotic cells or the apoptosis of CD68+ macrophages. Therefore, to examine whether rosiglitazone and
RvD1 act directly on macrophages and promote clearance of apoptotic cells, phagocytosis assays were performed in peritoneal macrophages (see fig. 3, Supplemental Digital Content 1, http://links.lww.com/ALN/B203). Stimulation with rosiglitazone or RvD1 increased phagocytic activity, equivalent to costimulation with rosiglitazone and RvD1 in m/m-derived cells (m/m rosi vs. m/m RvD1 vs. m/m rosi + RvD1: 2.191 ± 0.427 vs. 2.187 ± 0.434 vs. 2.401 ± 0.306 OD). In contrast, in db/db-derived cells, coadministration of RvD1 and rosiglitazone significantly increased zymosan uptake, whereas rosiglitazone or RvD1 alone did not alter phagocytic activity (db rosi + RvD1 vs. db rosi or RvD1: 2.234 ± 0.401 vs. 1.036 ± 0.363 vs. 1.256 ± 0.429 OD, P < 0.0001). These results support the hypothesis that RvD1 and PPARγ signaling act synergistically to enhance the phagocytic activity of macrophages in the inflammatory state.

**PPARγ-mediated Phenotype Shift of Macrophages from M1 to M2 Type Is Restored by Coadministration of RvD1 with Rosiglitazone in db/db Mice**

It has been demonstrated that PPARγ-regulated clearance of apoptotic cells is mediated mainly by activated macrophages. Recently, we reported that rosiglitazone attenuates mechanical hyperalgesia by altering macrophage polarity from M1 to M2 phenotype in C57BL6 mice. To evaluate whether rosiglitazone also alters macrophage polarity in the wounds of db/db mice, macrophages at the wound sites were counted after immunostaining for the pan-macrophage

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**Fig. 4.** Local infiltration of Gr-1+ polymorphonuclear neutrophils was decreased by rosiglitazone (rosi) coadministered with resolvin D1 (RvD1) in db/db mice. (A) Intact paws and incised sites on days 2 and 7 after incision. (B) Total number of Gr-1+ polymorphonuclear neutrophils in subcutaneous tissue was counted. Two-way ANOVA (interaction factor: \( F_{7,80} = 2.113, P = 0.0514 \)). The results are presented as mean ± SD. \(* P < 0.05, \ *** P < 0.001, \ **** P < 0.0001. \) Green = Gr-1; blue = 4',6-diamidino-2-phenylindole (DAPI); Scale bar = 100 μm.
marker, F4/80, with an M1-specific marker, iNOS, or an M2-specific marker, CD206 (fig. 6A). Consistent with a previous report that the induction of the M1 marker iNOS in macrophages is negatively regulated by PPARγ agonists,23 the number of F4/80*iNOS* M1 macrophages recruited in the early phase was significantly decreased by administration of rosiglitazone compared with vehicle in the incision sites of m/m mice on day 2 (m/m rosi vs. m/m vehicle: 261 ± 69 vs. 740 ± 344 cells/mm², P < 0.01) (fig. 6B). However, the number of F4/80*iNOS* M1 macrophages was not

**Fig. 5.** Apoptotic TdT-mediated dUTP nick-end labeling (TUNEL)+ cells were markedly decreased by rosiglitazone (rosi) coadministered with resolvin D1 (RvD1) in db/db mice at the incision site. (A) TUNEL+CD68+ DAPI+, TUNEL+DAPI+, and CD68+DAPI+ cells at the incised sites. (B) The number of TUNEL+CD68+ DAPI+ per area at the incision sites. Two-way ANOVA (interaction factor: $F_{7,96} = 2.806, P = 0.0106$) by separate one-way ANOVA with Tukey post hoc test for comparison at each time (day 2: $F_{7,48} = 7.587, P < 0.0001$; day 7: $F_{7,48} = 2.891, P = 0.0133$). (C) The number of TUNEL+DAPI+ cells per area at the incision sites. Two-way ANOVA (interaction factor: $F_{7,96} = 0.8077, P = 0.5829$) followed by Tukey post hoc test for comparing differences among groups. (D) The number of CD68+ DAPI+ cells per area at the incision sites. Two-way ANOVA (interaction factor: $F_{7,96} = 0.6218, P = 0.7368$). The results are presented as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001. Green = TUNEL; red = CD68; blue = DAPI. Scale bar, 100 μm. DAPI = 4',6-diamidino-2-phenylindole.
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altered by rosiglitazone in db/db mice (db rosi \textit{vs.} db vehicle: 751 ± 356 \textit{vs.} 726 ± 164 cells/mm²). Similarly, the number of F4/80+CD206+ M2 macrophages was not altered by rosiglitazone in db/db mice (db rosi \textit{vs.} db vehicle: 127 ± 97 \textit{vs.} 197 ± 121 cells/mm²), whereas rosiglitazone significantly increased the infiltration of F4/80+CD206+ cells at incision sites in m/m mice on day 2 (m/m rosi \textit{vs.} m/m vehicle: 563 ± 374 \textit{vs.} 127 ± 97 cells/mm², \textit{P} < 0.05) (fig. 6C). The total number of F4/80+ macrophages did not differ between m/m and db/db wounds, implying that the infiltration of monocytes from the circulation to the wounds was not impaired but that macrophage polarization, which has a pivotal role in the transition from inflammatory to wound healing phase, might be impaired in db/db mice.

Fig. 6. Phenotype shift of macrophages from M1 to M2 type was promoted by rosiglitazone (rosi) in m/m but not in db/db mice. (A) Infiltration of F4/80+iNOS+ M1 macrophages and F4/80+CD206+ M2 macrophages was evaluated on days 2 and 7. (B) The number of F4/80+iNOS+ and total F4/80 macrophages per area. Two-way ANOVA (F4/80+iNOS+: interaction factor: \textit{F}_{3,56} = 0.3627, \textit{P} = 0.7802; total F4/80+: interaction factor: \textit{F}_{3,56} = 0.08261, \textit{P} = 0.9692). (C) The number of F4/80+CD206+ and total F4/80 macrophages per area. Two-way ANOVA (F4/80+CD206+: interaction factor: \textit{F}_{3,56} = 1.046, \textit{P} = 0.3794; total F4/80+: interaction factor: \textit{F}_{3,56} = 1.122, \textit{P} = 0.3478). The results are presented as mean ± SD. *\textit{P} < 0.05, **\textit{P} < 0.01. Green = F4/80; red = iNOS or CD206; blue = DAPI; Scale bar = 100 μm. DAPI = 4',6-diamidino-2-phenylindole; iNOS = inducible nitric oxide synthase.
We examined whether the resolvin mediator of inflammation, RvD1, rescued impairment of PPARγ-mediated M2 phenotype shift of macrophages in db/db mice (fig. 7A). In m/m mice, RvD1 alone or coadministered with rosiglitazone did not alter the number of F4/80+ iNOS– M1 macrophages compared with rosiglitazone alone (fig. 7B). However, coadministration of RvD1 with rosiglitazone significantly increased F4/80+CD206+ M2 macrophages on day 2 compared with rosiglitazone alone (m/m rosi + RvD1 vs. m/m rosi: 637 ± 120 vs. 411 ± 188 cells/mm², P < 0.01) (fig. 7C). This suggests that RvD1 contributes to the polarization of M1 macrophages toward M2 type in m/m mice, without altering the number of total F4/80+ macrophages. In contrast to m/m mice, coadministration of RvD1 with rosiglitazone significantly decreased F4/80+ iNOS– M1 macrophages (db rosi + RvD1 vs. db rosi: 561 ± 181 vs. 168 ± 66 cells/mm², P < 0.0001) on day 2 (fig. 7B) and increased F4/80+CD206+ M2 macrophages (db rosi + RvD1 vs. db rosi: 137 ± 71 vs. 528 ± 98 cells/mm², P < 0.0001) on day 2 (fig. 7C) in db/db mice. The total number of F4/80+ cells was not altered by administration of RvD1. These data suggest that the phenotype shift of macrophages from M1 to M2 type during the course of postincisional inflammation is impaired in db/db mice but is effectively restored by coadministration of RvD1 with rosiglitazone. The number of iNOS–CD206+ macrophages was not significantly different between the groups or altered by treatment in m/m and db/db mice (see Supplementary Methods and fig. 4, Supplemental Digital Content 1, http://links.lww.com/ALN/B203). M1 macrophages are converted to an M2-like phenotype after encountering apoptotic PMNs; therefore, conversion from M1 to M2 phenotype might not be impaired once cells are committed to the M2 type.

Rosiglitazone Administered with RvD1 Alters Expression of Mediators Involved in Inflammation and Wound Healing

Tumor necrosis factor-γ has a pivotal role in neutrophil migration and the development of mechanical allodynia in inflammatory hyperalgesia. It has been reported previously that M1 macrophages predominantly produce nociceptive mediators, including TNF-α and iNOS. Consistent with the prolonged infiltration of Gr-1+ PMNs (fig. 4C), expression of TNF-γ mRNA (Tnf) was increased after incision in vehicle-injected hind paws of db/db compared with m/m mice (db vehicle vs. m/m vehicle: 22.372 ± 1.4157 vs. 10.252 ± 7.529, P < 0.05). Up-regulated TNF-α expression in db/db mice was significantly decreased by coadministration of RvD1 with rosiglitazone on day 7 (db vehicle vs. db rosi + RvD1: 22.372 ± 1.4157 vs. 7.543 ± 3.851, P < 0.01) (fig. 8), suggesting that RvD1/PPARγ signaling downregulates the proinflammatory mediator TNF-α, leading to attenuation of postincisional pain. We evaluated expression of Tgfb1, which is predominantly produced by M2 macrophages and promotes collagen synthesis and proliferation of fibroblasts during wound healing. The number of F4/80+ iNOS– M1 and F4/80+CD206+ M2 macrophages was not altered by administration of RvD1 or rosiglitazone in m/m mice on day 7 (fig. 7); therefore, the expression of TGF-β1 and TNF-α was not altered in mice receiving rosiglitazone and/or RvD1. However, gene expression of TGF-β1 was significantly increased by coadministration of rosiglitazone with RvD1 compared with rosiglitazone alone in db/db wounds (db rosi + RvD1 vs. db rosi: 4.598 ± 2.766 vs. 1.533 ± 0.906, P < 0.01), consistent with the increase in F4/80+CD206+ M2 macrophages by coadministration of RvD1 with rosiglitazone in db/db mice (fig. 7C).

We evaluated the effects of DMSO and 0.1% ethanol, used as solvents of rosiglitazone and RvD1, respectively, on inflammatory responses. In contrast to the effects of DMSO/0.1% ethanol on incision-induced paw edema and pain behavior (fig. 2), no changes in neutrophil infiltration (see fig. 5, Supplemental Digital Content 1, http://links.lww.com/ALN/B203), macrophage phenotype (see fig. 7, Supplemental Digital Content 1, http://links.lww.com/ALN/B203), or induction of TNF-α and TGF-β1 at the incision sites (see fig. 8, Supplemental Digital Content 1, http://links.lww.com/ALN/B203) were observed. This is consistent with the previous study in which subcutaneous injection of DMSO enhanced zymosan-induced edema up to 3 days after injection but did not affect the inflammatory response in formalin-induced or carrageenan-induced edema or in a model of burn edema in rats. Therefore, repeated subcutaneous administration of DMSO and 0.1% ethanol might enhance paw edema without altering the nociceptive profile and the population of immune cells in the course of incision-induced peripheral inflammation.

Discussion

We demonstrated that PPARγ-mediated analgesia was impaired in diabetic db/db mice, which was effectively restored by coadministration of RvD1. Restoration of PPARγ-mediated analgesia was associated with reduced infiltration of PMNs and apoptotic cells and accelerated macrophage polarization from M1 to M2 phenotype in incisional wounds of diabetic mice (fig. 9). Piercy et al. demonstrated that oral administration of rosiglitazone had no effect on the mechanical threshold in Zucker diabetic fatty rats although the latency to thermal stimulation was increased. We found that thermal latency was not altered by rosiglitazone in m/m and db/db mice. This discrepancy can be partly explained by the difference in genetic phenotype of the diabetic model or in the route of rosiglitazone administration. In addition, we speculate that the requirement for lipid mediators involved may be increased in incision-induced inflammation in diabetes.

The wound environment of diabetic patients is characterized by excessive inflammation in response to injury during the late phase of wound healing, which may prolong peripheral sensitization, leading to the development of postincisional hyperalgesia. We previously reported that transplantation of rosiglitazone-treated peritoneal macrophages into...
Fig. 7. Decreased infiltration of F4/80+CD206+ M2 macrophages was restored by coadministration of rosiglitazone (rosi) with resolvin D1 (RvD1) in db/db mice. (A) Infiltration of F4/80+ iNOS+ M1 macrophages and F4/80+CD206+ M2 macrophages was evaluated on days 2 and 7. (B) The number of F4/80+ iNOS+ and total F4/80+ macrophages. Two-way ANOVA (F4/80+ iNOS+: interaction factor: $F_{5,84} = 0.3126, P = 0.9041$; total F4/80+: interaction factor: $F_{5,84} = 0.7995, P = 0.5532$). (C) The number of F4/80+CD206+ and total F4/80+ macrophages. Two-way ANOVA (F4/80+CD206+: interaction factor: $F_{5,84} = 1.132, P = 0.4350$; total F4/80+: interaction factor: $F_{5,84} = 0.8349, P = 0.3498$). The results are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Green = F4/80; red = iNOS or CD206; blue = 4',6-diamidino-2-phenylindole (DAPI); Scale bar = 100 μm. iNOS = inducible nitric oxide synthase.
TNF-α and TGF-β1 expression was increased at the incision site in db/db mice on day 7 after incision compared with m/m mice.39 It has been reported that TNF-α inhibition in the cutaneous wounds of diabetic mice impaired cutaneous wound healing10 in db/db wounds (fig. 5B). However, infiltration of phagocytic CD68+ cells was not decreased in db/db mice. These results might be explained by the previous reports that M1 and M2 macrophages are highly efferocytic compared with M1 and presumably produce 12/15-lipoxygenase-derived proresolving lipid mediators including RvD1.22 Although proresolving mediators including RvD1 are present in subcutaneous fat of human wounds,60 and RvD1 is decreased in db/db mice,10 the peripheral actions of RvD1 after surgical procedures have not been explored. In addition, fat mass enlargement in adipose tissue of subcutaneous lesions is associated with the phenotypic changes of macrophage subsets.37 Therefore, we focused on the changes in the compartment of subcutaneous tissue in this study. However, we cannot exclude the possibility that immune cells in the other compartments such as the dermis and epidermis have a role in incision-evoked inflammatory responses.

It has been reported that cytokine dysregulation in response to bacterial infection is associated with persistent inflammation including PMN infiltration, which is reversed by TNF-α inhibition in the cutaneous wounds of db/db mice.38 A sustained increase in TNF-α impairs cutaneous wound healing with decreased collagen production in the wounds of diabetic mice.39 It has been reported that TNF-α accelerates PMN apoptosis,40 which is consistent with our data showing that the wounds of db/db mice exhibited high expression of TNF-α associated with a decrease in CD68+ TUNEL+ cells (fig. 5B). RvD1, which promotes efferocytosis by macrophages, suppresses TNF-α expression41; therefore, PMN apoptosis induced by TNF-α might be followed by efferocytosis promoted by RvD1 under nondiabetic conditions. Mirza et al.42 have reported that macrophage depletion in diphtheria toxin receptor-transgenic mice results in delayed reepithelialization, decreased collagen deposition, impaired angiogenesis, and reduced cell proliferation associated with increased production of TNF-α and decreased production of TGF-β1 in excised skin wounds. These findings suggest that local macrophages make a major contribution to production of TNF-α and TGF-β1 induced by incision during postoperative periods. However, we cannot exclude the possibility that local cells involved in wound healing, such as keratinocytes, fibroblasts, and endothelial cells, contribute to the complex signaling network involving numerous cytokines and growth factors during development of incision-induced inflammation.

Macrophages potentiate voltage-gated sodium channel currents through the TNF-α signaling pathway and further
downstream phosphorylation of p38 mitogen-activated protein kinase in dorsal root ganglia (DRG).\textsuperscript{43} Intraplantar injection of TNF-α evokes up-regulation of calcitonin gene–related peptide and nerve growth factor (NGF) and prostaglandin E2.\textsuperscript{44} Intraplantar injection of TNF-α is sufficient to induce mechanical hyperalgesia;\textsuperscript{45} therefore, we speculate that the sustained high levels of TNF-α at the incision sites of db/db mice directly exacerbate the development of postoperative hyperalgesia. As another possibility, Cheng \textit{et al.}\textsuperscript{15} reported increased expression of NGF and neuropeptides such as substance P and calcitonin gene–related peptide in DRG in db/db mice, consistent with the decrease in 50% mechanical threshold peak at 8 weeks of age. In the hind paw skin as well as in the DRG, NGF immunoreactivity was strongly detected in dermal cells compared with db/+ mice. In addition, intraperitoneal injection of anti-NGF decreased substance P expression in the DRG, suggesting that NGF is a pivotal mediator regulating profile changes in primary sensory neurons. Dermal immune cells including macrophages can express NGF and secreted NGF from peripheral nerve endings can also trigger neurogenic inflammation.\textsuperscript{46} Thus, increase in NGF in db/db mice might contribute to neuroimmune interaction evoked by dermal incision, leading to macrophage phenotype changes and mechanical allodynia. Further investigation is required to clarify the mechanism by which phenotypic changes in peripheral macrophages can cause the development of incision-induced mechanical hyperalgesia in diabetes.

Peroxisome proliferator–activated receptor-γ (PPARγ) activation in db/db mice was not sufficient to induce macrophage polarization to M2 macrophages. TNF-α promotes macrophage polarization to M1 type, and M1 macrophages in turn produce TNF-α,\textsuperscript{47} which is regulated mainly by activation of transcription factors, including nuclear factor (NF)-κB in macrophages. PPARγ signaling inhibits inflammatory responses by transrepression of NF-κB target genes including TNF-α,\textsuperscript{23} whereas RvD1 replaces the p65/p50 heterodimer of NF-κB with a p50/p50 homodimer for proteosomal NF-κB degradation,\textsuperscript{41} suggesting that rosiglitazone or RvD1 function cooperatively in the regulation of NF-κB. As another potential mechanism of synergic effects, it has been reported that RvD1 enhances microglial polarization to M2 type through interleukin-4-induced PPARγ activation in BV-2 cells, via phosphorylation of signal transducer and activator of transcription (STAT)6, an upstream signaling molecule of PPARγ.\textsuperscript{48} STAT6 inhibitor decreases PPARγ protein levels in the nucleus and DNA binding, suggesting the mechanism of the synergic effects of RvD1 and PPARγ, in which the activation of STAT6 by RvD1 might be required for translocation of PPARγ to the nucleus and its activation. As a potential target for RvD1-mediated analgesic effects, it has been reported that RvD1 directly inhibits intracellular Ca\textsuperscript{2+} increases induced by the activation of transient receptor potential channels in DRG neurons.\textsuperscript{49} Therefore, alternative analgesic pathways independent of macrophages cannot be excluded.

In summary, we demonstrated that local administration of the PPARγ agonist rosiglitazone attenuated incision-induced mechanical hyperalgesia through an RvD1-dependent mechanism in diabetic mice. RvD1-mediated resolution of inflammation is essential for PPARγ-mediated macrophage polarization to the M2 phenotype. We propose that resolution of inflammation followed by transition to a M2 macrophage phenotype is essential for the development of PPARγ-mediated analgesia in diabetes.

**Fig. 9.** Summary of resolvin D1 (RvD1)-dependent peroxisome proliferator–activated receptor-γ (PPARγ)-mediated analgesia in diabetes.
wound healing after surgery might be a potential therapeutic target for the treatment of postoperative pain development in diabetes.

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Competing Interests
The authors declare no competing interests.

Correspondence
Address correspondence to Dr. Hasegawa-Moriyama: Department of Anesthesiology and Critical Care Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakurakogoa, Kagoshima 890-8520, Japan. hase-mai@m3.kufm.kagoshima-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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