Proresolution Therapy for the Treatment of Delayed Healing of Diabetic Wounds

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Type 2 diabetes is a major health problem reaching epidemic proportions in developed countries, and its incidence is rapidly increasing in developing countries as well (1). It is a significant cause of mortality and is strongly associated with an increase in the risk of cardiovascular disease and cancer (2,3). Extensive research has shown that such clinical complications of type 2 diabetes could be attributed, in part, to low-grade chronic inflammation (4,5). Before the development of type 2 diabetes, changes in the metabolism of glucose and fatty acids activate innate immune responses that give rise to systemic insulin resistance, which in turn perpetuates and establishes a state of chronic inflammation (5). As a result, type 2 diabetes is associated with tissue dysfunction and injury, deficiencies in clearing microbial infections, and impaired wound healing (6).

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Received 24 May 2012 and accepted 5 August 2012.

DOI: 10.2337/db12-0684

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RESEARCH DESIGN AND METHODS

Animals and reagents. Leptin receptor–deficient male mice [B6.BKS(D)-LeprΔ/h3; db/db] and their wild-type controls (C57BL/6J) were purchased from The Jackson Laboratory at 8–12 weeks of age and maintained on normal chow. All procedures were approved by University of Louisville’s Institutional Animal Care and Use Committee (no. 11017). Resolvin D1 (RvD1) (7,8,17-trihydroxydocosanoids, markers of resolvin biosynthesis, was attenuated in diabetic wounds, and local application of RvD1 accelerated wound closure and decreased accumulation of apoptotic cells and macrophages in the wounds. These findings support the notion that diabetes impairs resolution of wound healing and demonstrate that stimulating resolution with proresolving lipid mediators could be a novel approach to treating chronic, nonhealing wounds in patients with diabetes. Diabetes 62:618–627, 2013

Normally, inflammation protects against infection and injury but must be resolved to prevent inadvertent tissue damage (7,8). The resolution of inflammation is accomplished by time- and site-specific generation of proresolving mediators that control both the magnitude and the duration of the inflammatory response (7,9). Among the mediators of resolution, resolvins have emerged as critical players that exert potent anti-inflammatory actions by blunting excessive polymorphonuclear neutrophil (PMN) infiltration into tissues and decreasing proinflammatory mediator production (7,10). Unlike other anti-inflammatory mediators that suppress inflammation, resolvins also promote macrophage phagocytosis of apoptotic cells and microbes and stimulate the clearance of phagocytes to enable return to homeostasis (7,11,12). These events are critical for resolution of inflammation because lingering apoptotic cells or phagocytes can undergo secondary necrosis causing unwarranted tissue damage (13,14). Hence, disruption of these endogenous pathways of resolution could give rise to chronic inflammation in type 2 diabetes. Nevertheless, diabetes-induced changes in the resolution of inflammation have not been extensively studied. Recent studies from our group and others have shown that proresolving mediators such as resolvins decrease adipose tissue inflammation and improve insulin sensitivity in murine models of type 2 diabetes (15,16). However, it is not known whether type 2 diabetes affects the resolution of inflammation and whether treatment with proresolving mediators would stimulate resolution and ameliorate clinical complications of type 2 diabetes, such as impaired wound healing.
Histology and immunohistochemistry. Wound tissues were formalin fixed, paraffin embedded, and sectioned. For analysis of apoptotic cells, macrophages, and Fpr2 expression, wounds were harvested at day 5, while granulation tissue formation was assessed at day 27. Deparaffinized sections were stained with hematoxylin-eosin. Wound macrophages were visualized by immunofluorescence microscopy using 5-μm-thick sections. For this, sections were blocked with Rodent Block M Blocking Reagent for 1 h after antigen retrieval and incubated with Alexa-Fluor 647–conjugated anti-CD68 (1:200) for 2 h. Slides were mounted with SlowFade Gold antifade reagent with DAPI. Fluorescent photographs were obtained using an EVOS fluorescence microscope.

Chromatography. Cell pellets were extracted with methanol containing deuterated internal standard (1 ng PGE2-d4), from WT and db/db mice. Analysis by liquid chromatography–tandem mass spectrometry. Wound tissue was collected at 72 h post-zymosan challenge. Macrophages were isolated from WT mice after dexamethasone administration (19). Our previous studies have shown that phagocytosis of apoptotic PMN and microbes is impaired in macrophages isolated from diabetic humans and rodents (21). To this end, we challenged db/db mice with the prototypic proresolving mediator RvD1. To this end, we challenged db/db mice with zymosan challenge in db/db mice than in WT mice, indicating that diabetes alters macrophage-mediated clearance of apoptotic PMN. Consistent with defective macrophage phagocytosis, 72 h after zymosan challenge, we found higher levels of FITC-labeled zymosan in the peritoneum of db/db mice relative to WT mice, despite no significant differences in total macrophage content (Fig. 1G).

Because proresolving mediators such as the resolvins have been shown to stimulate macrophage-mediated clearance of apoptotic PMN and microbes, we next assessed whether these defects could be corrected by stimulating resolution with the prototypic proresolving mediator RvD1. To this end, we challenged db/db mice with zymosan and allowed PMN to infiltrate to maximal levels. We then administered RvD1 at 32 h post-zymosan challenge and collected inflammatory exudates 16 h later (48 h after zymosan challenge; see scheme in Fig. 2A). We found that RvD1 administration consistently decreased the amount of PMN remaining in the peritoneum relative to vehicle-treated mice, indicating that RvD1 enhanced the clearance of these cells. As an additional test of our hypothesis that stimulating resolution improves macrophage phagocytosis in vivo, we assessed the clearance of apoptotic thymocytes in response to dexamethasone administration (19). As shown in Fig. 2B, apoptotic thymocytes accumulated to a significantly higher level in db/db mice than WT mice and coadministration of RvD1 markedly reduced the accumulation of apoptotic thymocytes in db/db mice. Thus, collectively, these observations suggest that treatment with RvD1 enhances the clearance of apoptotic cells by macrophages and thus restores defective resolution of inflammation.

Previous studies have shown that phagocytosis of apoptotic cells and opsonized microbes is impaired in macrophages isolated from diabetic humans and rodents (21–24).

RESINOSomes restore defective resolution of inflammation in acute peritonitis using WT and db/db mice. Despite having elevated resident peritoneal leukocytes (Fig. 1A), the extent of leukocyte infiltration in response to zymosan challenge was not different between WT and db/db mice after either 6 or 24 h (Fig. 1B). However, in WT mice, the number of total leukocytes decreased between 24 and 48 h, whereas leukocyte levels remained elevated in db/db mice. Our flow cytometric analyses of the leukocyte differential revealed that PMNs were rapidly cleared between 24 and 48 h in WT but not db/db mice (Fig. 1C and D). However, we found no significant differences in macrophage infiltration during the time course of acute peritonitis (Fig. 1E). As macrophage-mediated clearance of apoptotic PMN is a defining feature of active resolution (9), we assessed whether this process was altered in db/db mice. As shown in Fig. 1F, there was a greater accumulation of annexin V–positive PMN 48 h after zymosan challenge in db/db mice than in WT mice, indicating that diabetes alters macrophage-mediated clearance of apoptotic PMN. Consistent with defective macrophage phagocytosis, 72 h after zymosan challenge, we found higher levels of FITC-labeled zymosan in the peritoneum of db/db mice relative to WT mice, despite no significant differences in total macrophage content (Fig. 1G).
In addition, we and others have found that resolvins stimulate macrophage phagocytosis (7,12,20). Therefore, to test whether RvD1 rescues defective phagocytosis in diabetic macrophages, we isolated resident peritoneal macrophages from WT and db/db mice and assessed their ability to phagocytose IgG-opsonized zymosan. As shown in Fig. 3A, the phagocytic ability of macrophages isolated from db/db mice was significantly impaired compared with WT mice. We found that pretreatment with RvD1 at a concentration of just 0.1 nmol/L markedly stimulated phagocytosis by macrophages isolated from db/db mice (Fig. 3B). Because macrophage phagocytosis of both opsonized microbes and apoptotic cells requires PI3K (25) and this pathway is suppressed in diabetic mice (26), we evaluated whether the stimulation of phagocytosis by RvD1 was sensitive to PI3K blockade. Indeed, we found that the enhancement of phagocytosis by RvD1 was completely blocked in the presence of PI3K inhibitor, wortmannin (Fig. 3B).

Next, we sought evidence of a direct ligand receptor–dependent relationship between the effects of RvD1 and stimulated phagocytosis because recently the actions of RvD1 on macrophage phagocytosis were shown to involve the activation of G-protein–coupled receptor, formyl peptide receptor 2 (Fpr2) (also known as ALX) (20,27). Our results showed that stimulation of macrophage phagocytosis by RvD1 was blocked by a specific Fpr2 antagonist peptide, Trp-Arg-Trp-Trp-Trp-Trp (WRW4) (Fig. 3C) (28). To further examine the involvement of this receptor, we examined signaling events downstream of Fpr2 activation. Previous studies have shown that stimulation of phagocytosis by RvD1 is sensitive to pertussis toxin blockade, implicating coupling of Fpr2 to Gαi (20). Therefore, we measured the accumulation of a second messenger, cAMP, and found that in macrophages isolated from db/db mice, treatment with RvD1 was associated with a significant suppression of cAMP levels (Fig. 3D). These observations
are consistent with and lend further support to the involvement of Fpr2 in mediating the effects of RvD1 on macrophages. Taken together, these data indicate that RvD1 stimulates macrophage phagocytosis and thereby restores diabetic defects in resolution of acute inflammation.

Given that macrophage dysfunction is causally related to delayed wound healing in diabetes (23) and that this dysfunction can be rescued by RvD1, we examined whether stimulating resolution would ameliorate diabetes-induced defects in wound healing. For this, we evaluated the effects of RvD1 on the closure of cutaneous wounds in db/db mice. Consistent with previous reports, we found that in comparison with WT mice, wound closure was significantly delayed in db/db mice (Fig. 4A and B) (17). We then determined whether this delayed wound healing is associated with alterations in the resolvin biosynthetic pathway, using

FIG. 2. Resolvin D1 restores resolution of acute inflammation and promotes the clearance of apoptotic cells in obese diabetic mice. A: Schematic of treatment protocol of mice challenged with zymosan and then treated with RvD1 and leukocyte differentials (n = 8–9). B: Quantification of apoptotic cells in the thymus of WT and db/db mice challenged with dexamethasone (15 mg · kg⁻¹) and treated without or with RvD1 (1 μg) (n = 3–4). Top panel: Representative images of TUNEL staining per high power field (HPF), scale bars = 50 μm. Sections were counterstained with methyl green. Data are means ± SEM. *P < 0.05 by one-way ANOVA (B) or Student t test (A). (A high-quality digital representation of this figure is available in the online issue.)
an LC-MS/MS approach. These measurements showed that despite similar levels of the resolin biosynthetic precursor DHA (free, unesterified) in the wounds of WT and db/db mice, levels of monohydroxydocosanoids, which are markers of proresolving lipid mediator biosynthesis (resolvins, protectins, and maresins) (10), were lower in db/db mice (Fig. 4C).

To test the therapeutic efficacy of stimulating resolution in treating diabetic wounds, we applied RvD1 to cutaneous wounds of db/db mice. In this experiment, silicone splints were used to prevent skin contracture and to more closely monitor tissue growth (17). As shown in Fig. 5A, local delivery of RvD1 markedly enhanced the closure of diabetic wounds, and after 8 days of application the wound diameter was significantly smaller in RvD1-treated than in vehicle-treated animals. Consistent with improved wound regeneration, our histological analysis revealed that RvD1-treated wounds had more granulation tissue formation (Fig. 5B). Next, we assessed the expression of RvD1 receptor, Fpr2, in nondiabetic and diabetic wounds. Histological analysis of this receptor indicated that while it was abundant in the wounds of both WT and db/db mice, the expression of Fpr2 was significantly decreased in the wounds of db/db mice compared with WT mice (Fig. 5C). Overall, these results suggest that both altered biosynthesis and decreased Fpr2 expression likely underlie diabetes-induced impairment of resolution and wound healing, although these deficiencies can be overcome by exogenous RvD1 treatment.

Given that treatment with RvD1 decreased the accumulation of apoptotic thymocytes and that accumulation of apoptotic cells is a feature of unhealed diabetic wounds (23), we next assessed whether local delivery of RvD1 would modulate the apoptotic cell burden in diabetic wounds. We found that the abundance of apoptotic cells was significantly decreased in RvD1-treated wounds relative to vehicle treatment, as assessed by histological analysis (TUNEL; Fig. 6A and C). Lastly, we also observed that the number of CD68+ macrophages, which also accumulate in wounds of diabetic mice, was significantly decreased by RvD1 treatment at 5 days postwounding (Fig. 6B and C) (29). Overall, these results are consistent with our in vitro and acute in vivo studies demonstrating that RvD1 enhances macrophage phagocytosis and clearance of apoptotic cells and suggest that this could in part contribute to the enhanced wound healing observed in RvD1-treated mice.

**DISCUSSION**

The results of this study demonstrate that type 2 diabetes alters the resolution of inflammation and that these alterations can be acutely corrected by stimulating resolution with proresolving lipid mediator RvD1. In addition, our data indicate that RvD1 also restores diabetic defects in macrophage phagocytosis, which could decrease the accumulation of apoptotic/necrotic cells and microbes in chronically inflamed tissues. We found that stimulating resolution with RvD1 enhanced closure of diabetic wounds and this was also associated with decreased burden of both apoptotic cells and macrophages. Thus, RvD1 could potentially be used as a novel agent to promote wound healing in diabetic patients.

A large body of research demonstrates that inflammatory signaling pathways are chronically activated in obesity and diabetes, and that adipose tissue expansion promotes infiltration of mononuclear cells that secrete proinflammatory mediators and sustain insulin resistance (4,5). In healthy tissue, pathogen invasion or tissue injury elicits prompt infiltration of leukocytes that limit the injurious stimulus. Infiltrating PMNs, however, rapidly undergo apoptosis, and they must be cleared by macrophages to prevent inadvertent tissue damage (9). In addition, macrophages also phagocytose microbes and promote their removal. These events are critical for active resolution of inflammation and are regulated by endogenous proresolving mediators (10). Thus, we first examined whether chronic inflammation in obesity and diabetes could result from
failed resolution. For this, we used an acute peritonitis model because it allows for the time-dependent analysis of leukocyte infiltration, apoptosis, and phagocyte clearance. Our results showed that, rather than elevated leukocyte infiltration as might be expected, the macrophage-mediated removal of apoptotic cells and zymosan was impaired in diabetic mice, which further illustrates that specifically targeting these cellular events in resolution could have beneficial effects on chronic inflammation. Importantly, we found that therapeutic administration of proresolving lipid

FIG. 4. Diabetes impairs wound healing and metabolism of DHA. A: Progressive changes in cutaneous wound closure in WT and db/db mice. B: Quantitative analysis of wound closure in WT and db/db mice (n = 5). C: Quantification of DHA and downstream metabolites, 17-HDHA, 14-HDHA, and 4-HDHA by liquid chromatography–tandem mass spectrometry analysis using MRM in cutaneous wounds isolated from WT and db/db mice at day (d) 5 (n = 4). The MRM transitions that were used are as follows: DHA, 327 > 283; 17-HDHA, 343 > 147; 14-HDHA, 343 > 161; and 4-HDHA, 343 > 101. Schematic of downstream mediators generated from DHA is also shown. Data are means ± SEM. *P < 0.05 by two-way ANOVA (B) or Student t test (C). (A high-quality digital representation of this figure is available in the online issue.)
mediator, RvD1, decreased PMN accumulation in the peritoneum. Although we administered RvD1 at the time of maximal PMN infiltration, we cannot rule out the possibility that decreased PMN accumulation in response to RvD1 treatment could be due to decreased infiltration. Thus, we next turned to a distinct model of phagocyte-mediated clearance of apoptotic cells that does not depend on infiltrating cells but, rather, on the apoptosis of resident cells (19). The results of these studies demonstrated that RvD1 decreases the accumulation of apoptotic thymocytes in

FIG. 5. RvD1 accelerates wound closure and granulation tissue formation in db/db mice. A: Representative images of vehicle (0.1% ethanol in saline) and RvD1-treated (100 ng per day [d] per wound) splinted wounds in db/db mice. Lower panel: Quantitative analysis of wound closure in db/db mice treated without or with RvD1, with the structure of RvD1 shown in the inset (n = 5–9). B: Assessment of granulation tissue area in vehicle or RvD1-treated wounds in db/db mice. Representative histological sections of hematoxylin-eosin staining (top panels, scale bar = 1,000 μm; lower panels, scale bar = 200 μm). Lower panel: Quantification of granulation tissue area (day 27; n = 5). C: Histological analysis of RvD1 receptor, Fpr2, in wounds isolated from WT and db/db mice at day 5 (n = 5). Sections were counterstained with hematoxylin-eosin; scale bars = 200 μm (left two panels) and 50 μm (right two panels) for each group. Quantification of Fpr2 receptor density is shown as pixels per low power field (LPF). Data are means ± SEM. *P < 0.05 by two-way ANOVA (A) or Student t test (B and C). (A high-quality digital representation of this figure is available in the online issue.)
response to dexamethasone administration. These in vivo data strongly suggest that RvD1 improves macrophage-mediated clearance in diabetes and highlight the importance of the metabolic environment in diabetes in altering innate immune responses required for resolution.

An important biological action of resolvins is that they stimulate macrophage phagocytosis and emigration from tissues. We and others have previously demonstrated that resolvins, such as RvD1 and RvD2, enhance the phagocytosis of apoptotic cells, opsonized zymosan, and live bacteria (11,12,30). Importantly, stimulation of macrophage phagocytosis by RvD1 is mediated by Fpr2 (20,27). It has also been shown before that macrophages isolated from both diabetic humans and rodents have impairments in their ability to undergo phagocytosis (21,24). This defect extends to uptake of both apoptotic cells and microbes and has been demonstrated in several distinct rodent models of both type 1 and type 2 diabetes, including db/db, ob/ob, NOD, and streptozotocin-induced diabetes in mice (22,24,26,31). Thus, as a direct test of the hypothesis that RvD1 enhanced phagocyte-mediated clearance, we assessed its actions on primary macrophages isolated from db/db mice. We found that RvD1 enhanced diabetic macrophage phagocytosis in a receptor-dependent manner, suggesting that the proresolving role of RvD1 in vivo could be attributed in part to its targeted actions on macrophages.

Delayed wound healing is one of the most prominent clinical manifestations of type 2 diabetes. Current management of diabetic wounds is focused primarily on debridement, off-loading, antibiotic therapy, and in some cases, surgical revascularization (e.g., angioplasty and bypass) (6). Even with therapeutic management, diabetes still accounts for >60% of nontraumatic amputations of the lower limb, and nearly one-third of diabetic foot ulcers require surgery (1,32). Thus, novel therapeutics aimed at controlling inflammation, reducing infection, and improving wound closure are urgently needed. Deregulated inflammation impairs the normal wound-healing response in diabetes, which is associated with increased susceptibility to soft-tissue infection despite accumulation of leukocytes. In fact, diabetic wounds have elevated levels of both PMN and macrophages and express higher levels of proinflammatory cytokines (29). Macrophage dysfunction is central to altered healing of diabetic wounds. This leads to failed clearance of apoptotic cells and microbes and gives rise to tissue necrosis (23). The results of the current study demonstrate that RvD1 enhances wound closure in diabetic mice and that this is associated with decreased accumulation of apoptotic cells and macrophages in the wounds. The beneficial effects at RvD1 on wound healing observed here are likely to be of high clinical importance because reducing wound-healing time would reduce susceptibility to infection. Moreover, the enhancement of diabetic macrophage phagocytosis by RvD1 suggests that local delivery of resolvins could also combat existing wound infection as an adjunctive therapy to antibiotics.

FIG. 6. Apoptotic cell and macrophage levels are decreased in diabetic wounds treated with resolvin D1. A: Representative images of TUNEL staining in db/db wounds treated without or with RvD1 (day 5; n = 4/group). Scale bars = 200 μm (left panel) and 50 μm (right panel). B: Immunofluorescence imaging of CD68+ macrophages in db/db wounds treated without or with RvD1 and harvested at day 5. Scale bars = 200 μm (upper panel) and 50 μm (lower panel) for each group. C: Quantification of TUNEL+ cells and CD68+ cells in db/db wounds treated without or with RvD1 (n = 4) per high power field (HPF). Data are means ± SEM. *P < 0.05 by Student t test. (A high-quality digital representation of this figure is available in the online issue.)
Indeed, recent studies have demonstrated that resolvins lower the threshold for antibiotic therapy in live infection, and we have previously shown that resolvins enhance leukocyte-mediated bacterial killing (11,12). Moreover, even though the current study suggests that macrophages may be the primary targets of RvD1, we have previously demonstrated that RvD1 also has direct actions on human epidermal keratinocytes, and thus RvD1 could have multiple cellular targets within wounds (33). Further studies will be required to interrogate fully the prohealing roles for resolvins.

Previous studies have documented that n-3 polyunsaturated fatty acids (PUFAs) improve metabolic parameters in obese and diabetic mice. In particular, either dietary supplementation or genetic manipulation (fat-1 transgenic) to increase omega-3 PUA levels has been shown to be associated with improved whole-body insulin sensitivity and increased flux through proresolving lipid mediator biosynthetic pathways (16,34,35). Moreover, we have recently shown that RvD1 enhances glucose tolerance and decreases macrophage accumulation in hypertrophied adipose tissue in db/db mice (15). The results of the present studies extend these findings by illustrating that proresolving lipid mediators improve macrophage function and clinically relevant end points (i.e., wound healing). Interestingly, we found that levels of free, unesterified DHA were similar in wounds isolated from non-diabetic and diabetic mice but that biosynthetic intermediates in proresolving lipid mediator pathways were markedly decreased. This result is consistent with recent findings by another group, which also reported deficient levels of 14-HDHA and another DHA-derived mediator, 14S,21R-dihydroxyDHA, in diabetic wounds (36). Given that clinical trials with n-3 PUFAs have not yielded conclusive beneficial effects in humans with diabetes (37), the findings of the current study suggest that alterations in downstream metabolism of DHA might contribute to the discrepancies between rodent and human studies and that more targeted therapeutics with proresolving lipid mediators such as resolvins may be more efficacious than dietary supplementation with n-3 PUFAs.

In summary, the results of the current study demonstrate that resolution of inflammation is altered in type 2 diabetes and that defective macrophage-mediated resolution could be restored by proresolving lipid mediator RvD1. Notably, local delivery of RvD1 enhanced wound closure in diabetic mice, suggesting that stimulating resolution has beneficial functional outcomes. As resolvins are currently in phase III clinical trials for the treatment of other inflammatory pathologies (i.e., dry eye), the results of this study could be readily translated into clinical therapy for accelerating wound healing in patients with diabetes. The findings of the current study also have wide implications for developing future strategies for the treatment of other diabetes complications and several chronic autoimmune and cardiovascular diseases associated with unresolved chronic inflammation (8).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL106173 (to M.S.) and P20RR024489 (Diabetes and Obesity Center) to A.B. and M.S.). The study was also supported by U.S. Department of Defense Grant BAA10-1 (to A.B. and M.S.).

No potential conflicts of interest relevant to this article were reported.

Y.T., M.J.Z., and J.H. designed and carried out experiments, analyzed data, and contributed to the writing of the manuscript. M.K. carried out experiments and analyzed data. A.B. designed experiments and contributed to the writing of the manuscript. M.S. planned the project, designed experiments, analyzed data, and wrote the manuscript. M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Dr. Lucy Norling and Ms. Stefania Bena (William Harvey Research Institute, London, U.K.) for assistance with Ppr2 staining.

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