Wound Inflammation in Diabetic ob/ob Mice

Functional Coupling of Prostaglandin Biosynthesis to Cyclooxygenase-1 Activity in Diabetes-Impaired Wound Healing

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This study focused on the regulation of prostaglandin (PG) production in diabetes-impaired wound tissue. Cyclooxygenase (COX)-1 and -2 expression and activity were severely dysregulated in chronic wounds of diabetic ob/ob mice. Those wounds were characterized by a reduced expression of COX-1 and the presence of strongly elevated levels of COX-2 when compared with conditions observed in healthy animals. Resolution of the diabetic and impaired wound-healing phenotype by systemic administration of leptin into ob/ob mice increased COX-1 expression in wound margin keratinocytes and decreased COX-2 expression in inner wound areas to levels found in wild-type animals. Notably, improved wound healing was characterized by a marked increase in PGE₂/PGD₂ biosynthesis that colocalized with induced COX-1 in new tissue at the margin of the wound. COX-2 expression did not significantly contribute to PGE₂/PGD₂ production in impaired wound tissue. Accordingly, only late wound tissue from SC-560-treated (selective COX-1 inhibitor) but not celecoxib-treated (selective COX-2 inhibitor) ob/ob mice exhibited a severe loss in PGE₂, PGD₂, and prostacyclin at the wound site, and this change was associated with reduced keratinocyte numbers in the neo-epithelia. These data constitute strong evidence that a dysregulation of COX-1-coupled prostaglandin contributes to diabetes-impaired wound healing. Diabetes 54: 1543–1551, 2005

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Wound Inflammation is driven by a variety of mediators that are tightly controlled in space and time (6,18). Besides the typical protein-type mediators, resident and invading cells in wound tissue communicate the initiation and resolution of the inflammatory process through the production of a variety of eicosanoids (19,20). Especially the prostaglandins (PGs), which are derived by enzymatically coupled biosynthetic pathways involving phospholipase A₂, cyclooxygenase (COX), and PG synthase isoenzymes,
are discussed to be central to these processes (21,22). In line with this, the COX-2 isozyme is rapidly induced under inflammatory conditions (21). However, COX-1–coupled but not COX-2–coupled PG synthesis is pivotally involved in undisturbed skin repair. Accordingly, wound PGE2/PGD2 biosynthesis was significantly reduced after selective inhibition of COX-1 but not COX-2 activity and resulted in an impairment of keratinocyte proliferation in healthy mice (22). Here we report evidence for a marked loss of PG biosynthesis in diabetes-impaired wound tissue of ob/ob mice in the presence of strongly elevated COX-2 levels. Moreover, improvement of skin repair after systemic leptin administration in ob/ob mice was associated with significantly increased PG levels that were synthesized by a COX-1–coupled pathway located at the margins of the wound. Thus, this study provides evidence that dysregulation of COX-1–coupled but not a COX-2–coupled PG biosynthesis pivotally contributes to strongly impaired repair conditions in diabetic ob/ob mice.

**RESEARCH DESIGN AND METHODS**

Female C57BL/6J (wild-type), C57BL/6J-db/db, and C57BL/6J-ob/ob mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained under a 12-h light/12-h dark cycle at 22°C until they were 8 weeks of age. At this time, they were caged individually, monitored for body weight, and wounded as described below.

**Treatment of mice.** Murine recombinant leptin (Calbiochem, Bad Soden, Germany) was injected intraperitoneally once a day at 8:00 A.M. (2 µg/g body wt) in 0.5 ml PBS per injection for 13 days. Control mice were treated with PBS alone. SC-560 (Witega, Berlin, Germany) or celecoxib (Celebrex; Pfizer, Karlsruhe, Germany) were administered orally twice daily by gastrogavage to db/db mice. Wounding of mice was performed as described previously (23,24). Briefly, mice were anesthetized. Subsequently, six full-thickness wounds (5 mm diameter, 3–4 mm apart) were made on the back of the mice by excising the skin and the underlying panniculus carnosus. The wounds were allowed to dry to form a scab. An area of 7–8 mm in diameter, which included the granulation tissue and the complete epithelial margins, was excised at the indicated time points for analysis. All animal experiments were carried out according to the guidelines and were approved by the local ethics animal review board.

**RNA isolation and RNAse protection analysis.** RNA isolation and RNase protection assays were carried out as described previously (24,25). For the RNase protection assay, DNA probes were cloned into the transcription vector pBluescript II KS (+) (Stratagene, Heidelberg, Germany) and linearized. An antisense transcript was synthesized in vitro using T3 RNA polymerase and [32P]-UTP (800 Ci/mmol). Total RNA samples (20 µg) were hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNases A and T1 for 1 h at 30°C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% acrylamide/8 mol/l urea gels and analyzed using a PhosphorImager (Fuji, Straubenhardt, Germany). The murine cDNA probes were cloned using RT-PCR. The probes corresponded to nt 1082–1946 (for COX-1, NM34114), nt 796–1063 (for COX-2, M64291), nt 223–475 (for membrane-associated PG synthase 1, AB041997), nt 792–947 (for cytosolic PG synthase, AK007198), nt 416–643 (for hematopoietic PG synthase, D82072), nt 421–656 (for prostacyclin synthase, NM008968.1), nt 13381–13583 (for thromboxane synthase, NM011539.1), nt 425 (exon 1) to 170 (exon 2) (for lysozyme M, M21047), and nt 163–517 (for GAPDH, NM002046).

**Immunohistochemistry and determination of neo-epithelial keratinocyte numbers.** Mice were wounded as described above. Animals were killed at day 13 after injury. Complete wounds were isolated from the back, bisected, fixed in paraformaldehyde/PBS (4% wt/vol), and frozen in tissue-freezing medium. Then, 6-µm sections were subsequently analyzed using immunohistochemistry as described (23). Antisera against COX-1, COX-2 (Santa Cruz, Heidelberg, Germany), macrophage-specific F4/80 antigen (Serotec, England), and keratin were used for immunodetection. For determination of keratinocyte numbers, sections were counterstained with Mayer's hematoxylin solution (ApplChem, Darmstadt, Germany). Keratinocyte cell numbers were subsequently determined in a defined wound area (0.5 mm).

**RESULTS**

**COX-1 and COX-2 expression and PGE2/PGD2 biosynthesis in diabetes-impaired wound healing.** In this study, we investigated the role of COX isoenzymes in diabetes-impaired wound healing in ob/ob and db/db mice. Both mouse models are characterized by severe type 2 diabetes (12) and suffer from disturbed wound-healing conditions (10,11,17). Nonwounded skin of C57BL/6J wild-type mice as well as skin of ob/ob and db/db mice was characterized by a strongly expressed COX-1 (7,200 ± 1,002 Phospholimager counts/20 µg RNA), whereas COX-2 expression (371 ± 52 Phospholimager counts/20 µg RNA) was nearly absent (Fig. 1A and B, ctrl skin). Upon wounding, COX-1 expression declined but COX-2 mRNA was markedly increased at this stage in normal and diseased animals (Fig. 1A and B). However, at late time points of repair (13d wound), we recognized a severe dysregulation of COX-1 and COX-2 expression in impaired healing conditions. COX-1 mRNA expression was markedly reduced in chronic wounds, whereas COX-2 was dramatically overexpressed at the mRNA level compared with normal healing conditions (Fig. 1A and B, right panels, 13d wound). Induction of COX-1 and -2 (Fig. 1A and B) was normalized to GAPDH mRNA expression. The shown differences in GAPDH mRNA levels (lower panels) were not due to unequal loading, because GAPDH (Fig. 1C) and other “housekeeping” genes (data not shown) are differentially regulated during normal and impaired skin repair. Staining of directly neighbored serial sections revealed...
that COX-2 protein was predominantly expressed in infiltrating macrophages in diabetes-impaired wounds isolated from ob/ob mice (Fig. 4B and C). However, COX-2 was not expressed in all wound macrophages, and COX-2 was also present in nonmacrophage cells at the wound site (Fig. 4C). Although present in wounds of leptin-treated ob/ob mice (Fig. 4D), we could not detect COX-1 protein in late wound tissue of diseased animals by immunohistochemistry (data not shown).

It is well established that systemic treatment of leptin-deficient ob/ob mice with recombinant leptin resulted in resolution of both the diabetic and impaired healing phenotype (11,17). Thus, ob/ob mice were injected intraperitoneally with recombinant leptin (2 μg/g body wt, once a day) for 13 days. As shown in Fig. 2A, we found high serum leptin levels 3 h after injection. The diabetic phenotype of leptin-injected ob/ob mice was resolved, because hyperinsulinemia (Fig. 2B) as well as blood glucose (Fig. 2C) were rapidly adjusted to normal. Moreover, mice revealed a significant loss of body weight (Fig. 2D) and an improved healing (Fig. 2E). Healing was assessed by counting scab-covered or scab-free wound areas on the backs of the mice. The loss of complete scabs represents a simple readout for wound re-epithelialization.
Next, we recognized that impaired healing conditions at late stages of repair (day 13 after wounding) were associated with significantly reduced expression of COX-1 (Fig. 3A) in the presence of a strongly overexpressed COX-2 (Fig. 3B) at the mRNA level. However, readjustment of both diabetic phenotype and chronic healing conditions after leptin administration (Fig. 2) was paralleled by a marked and significant re-increase of COX-1 (Fig. 3A) and a dramatic downregulation of COX-2 (Fig. 3B) mRNA. Accordingly, we found COX-2 protein to be predominantly expressed in macrophages present in chronic wound tissue (Fig. 4B and C). Moreover, leptin treatment led to a complete loss of COX-2–specific protein signals during improved skin repair (Fig. 4A). It is important to note that the observed downregulation of COX-2 was not due to a general reduction of macrophage influx during improved repair. We assessed the presence of macrophages at the wound site using lysozyme M mRNA, which is constitutively expressed in macrophages. As shown in Fig. 4A–C, COX-2 protein clearly diminished in the presence of unaltered numbers of macrophages. In line, we could not detect immunopositive COX-1 signals in chronic wounds.
(data not shown), although the COX-1 isoenzyme was strongly expressed in keratinocytes of the neo-epithelium in wounds from leptin-treated ob/ob mice (Fig. 4D).

COX-1 and -2 isoenzymes have to be coupled with various PG synthases to allow the final formation of different PG molecules (27). mRNA for membrane-associated PGE synthase 1, cytosolic PGE synthase, and hematopoietic PGD synthase was abundantly expressed, whereas expression of prostacyclin synthase and thromboxane synthase was present but weak in late wound tissue (Fig. 3C). However, expression levels of all examined PG synthases were not altered in impaired healing conditions (Fig. 3C). Finally, we determined wound PGE$_2$/PGD$_2$ levels by LC/MS/MS as an index of the overall wound COX/PG synthase enzyme activity in late wound tissue of PBS- and leptin-treated ob/ob mice (Fig. 3D). Remarkably, PGE$_2$/PGD$_2$ levels were significantly reduced in diabetes-impaired wound tissue isolated from PBS-treated mice when compared with improved healing conditions in leptin-administered animals in the presence of highly elevated expression levels of COX-2 (Fig. 3B and D). Thus, it is important to note that wound PGE$_2$/PGD$_2$ levels clearly followed the expression kinetics of the COX-1 isoenzyme at the wound site after leptin treatment of the animals (Fig. 3A and D).

**Wound margin tissue represents the predominant source of PGE$_2$/PGD$_2$ biosynthesis at the wound site.**

As a next step, we now analyzed the expression of COX-1 and COX-2 as well as PGE$_2$/PGD$_2$ concentrations in distinct compartments of wound tissue. Tissue was taken from 13-day disturbed (PBS-treated ob/ob mice) and improved (leptin-treated ob/ob mice) wounds and divided into “wound margin tissue” (which contained part of the nonwounded epidermis and dermis and, more importantly, the complete developing wound margin epithelia) and the “inner wound” (which contained the complete developing granulation tissue consisting of macrophages, fibroblasts, and endothelial cells). We found COX-1 to be predominantly expressed at the wound margins in disturbed as well as improved healing conditions. Nevertheless, improved healing after leptin administration was characterized by a significant doubling of COX-1 expression in wound margin tissue (Fig. 5A, upper panels). By contrast, COX-2 expression was clearly restricted to disturbed healing conditions in ob/ob mice and was predominantly located in the inner wound compartment. However, leptin-improved wound tissue was characterized by a complete downregulation of COX-2 expression (Fig. 5A, lower panels). Induction of COX-1 and -2 (Fig. 5A) was normalized to individual GAPDH mRNA levels. Moreover, a spatial coupling at the enzyme activity level could be established using LC/MS/MS analysis of PGE$_2$/PGD$_2$ production in separated wound compartments (Fig. 5B). Leptin-mediated increase in COX-1 expression at the wound margins was paralleled by a strong increase (about threefold) in PGE$_2$/PGD$_2$ levels (Fig. 5B). The leptin-driven increase in PGE$_2$/PGD$_2$ levels at the wound margins must be attributed to COX-1, because we could not detect any COX-2 expression at the wound site after leptin treatment (Fig. 5A, lower panels). It is important to note that PGE$_2$/PGD$_2$
concentrations in granulation tissue, despite the observed dramatic downregulation of overexpressed COX-2 (Fig. 5A), changed only moderately compared with the marked upregulation of PGE<sub>2</sub>/PGD<sub>2</sub> at the wound margins.

**COX-2 does not contribute to prostaglandin biosynthesis in diabetes-impaired wound healing.** Next, we used a pharmacological approach to confirm a COX-1–restricted production of PG in the presence of elevated levels of COX-2 during impaired skin repair. To differentiate COX-1– and COX-2–specific functions during repair, we treated ob/ob mice with the selective COX-1 inhibitor SC-560 and the selective COX-2 inhibitor celecoxib, respectively. Animals received oral doses (7.5 mg · kg<sup>-1</sup> · 12 h<sup>-1</sup>) from day 5 after wounding to prevent an inhibition of early wound inflammation, which is known to be pivotal to initiate the healing process (6). First, we assessed that both drugs had no long-term influence on metabolic parameters that are regulated by leptin in ob/ob mice. An 8-day treatment of the animals with SC-560 as well as celecoxib did not alter body weight (mock: 52 ± 8 g; SC-560: 51 ± 6 g; celecoxib: 52 g ± 4 g) or blood glucose levels (mock: 322 ± 113; SC-560: 301 ± 123; celecoxib: 296 ± 74).

We have established LC/MS/MS (for PGE<sub>2</sub>) and enzyme immunoassay (for PGD<sub>2</sub>) to determine both PGE<sub>2</sub> and PGD<sub>2</sub> subsets independently. Moreover, we have extended our analysis to additional PG subsets, thromboxane A<sub>2</sub> and prostacyclin (PGI<sub>2</sub>), because we had to exclude the possibility that COX-2 might potentially drive synthesis of PG molecules different to PGE<sub>2</sub>/PGD<sub>2</sub>. It is important to note that only selective inhibition of COX-1 by SC-560 led to markedly reduced levels of PGE<sub>2</sub>, PGD<sub>2</sub>, and 6-keto PGF<sub>1α</sub> (a stable metabolite derived from PGL<sub>2</sub>) in homogenates of total wound tissue (Fig. 6A, B, and D, left panels) and nonwounded back skin (Fig. 6A, B, and D, right panels). Reduction of 11-dehydro thromboxane B<sub>2</sub> (a stable metabolite derived from thromboxane A<sub>2</sub>) was also restricted to SC-560 treatment; however, the reduction failed to reach significance in wound tissue (Fig. 6C, right panel). Moreover, PGD<sub>2</sub> (Fig. 6B, right panel) and PGI<sub>2</sub> (Fig. 6D, right panel) were also only significantly reduced in inner wound tissue after COX-1 inhibition, although we had determined a strong COX-2 expression in these wound compartments (Figs. 4A and 5A). Because celecoxib has to be available at the wound site to inhibit COX-2 activity, we determined the availability of the drug 3 h after administration using LC/MS/MS. This was important, because we had to exclude the possibility that the absence of celecoxib at the wound site might mimic the failure of the drug to reduce wound PG synthesis. As shown in Fig. 7, blood serum levels for celecoxib were high, and the drug was also available in sufficiently high concentrations in wound margin and inner wound tissue.

**Inhibition of COX-1 interferes with formation of the neo-epithelium.** Finally, we determined keratinocyte cell numbers in the weak neo-epithelia that had formed in diabetes-impaired wounds at late stages of repair. For this purpose, we examined 13-day wound sections from ob/ob mice that have been treated with SC-560 or celecoxib, respectively. The sections were counterstained with hematoxylin, and the number of neo-epithelial keratinocytes within a defined wound area (0.5 mm) was counted (Fig. 8, upper right panel). Not unexpected, only selective inhibition of COX-1 by SC-560 moderately reduced keratinocyte cell numbers in impaired wounds (Fig. 8, left panel), suggesting that COX-1–coupled PG biosynthesis is centrally related to a normal regeneration process of skin tissue.

**DISCUSSION**

Wound inflammation pivotaly orchestrates intercellular movements and communication within the regenerating tissue. Wound inflammation is characterized by a cellular infiltrate (granulocytes, macrophages) and a locally restricted synthesis of a variety of inflammatory mediators (cytokines, chemokines, eicosanoids, nitric oxide, etc.). Immune cells initiate the healing process by release of a
variety of cytokines and growth factors (6,18,28). However, wound inflammation is janus-faced, and an uncontrolled and augmented inflammatory process is associated with chronically impaired healing conditions in mice and also in humans (8-11).

In this study, we have focused on the role of COX-driven eicosanoid biosynthesis in diabetes-impaired and leptin-improved skin repair in mice. This aspect was important to us for two distinct reasons. First, both COX-1 and -2 isoenzymes are directly implicated in inflammatory processes in different animal models of inflammation and evidenced to serve pro-inflammatory as well as anti-inflammatory actions (20). Second, a COX-1-coupled PG biosynthesis located in new tissue at the wound margins has been shown to represent an essential prerequisite for skin repair in healthy mice by driving a normal epithelial movement during the healing process (22).

Not unexpected, we found a dysregulation of COX-1 and -2 expression during skin repair in diabetic mice. Whereas COX-1 was subsequently reduced during impaired healing, we assessed a very strong induction of COX-2 upon injury. Because early wounding is characterized by a release of pro-inflammatory mediators (6,18), this finding was consistent with the general inducibility of COX-2 by these mediators (21). Moreover, COX-2 expression also remained dramatically increased during late repair and thus paralleled the sustained presence of inflammatory cytokines in these wounds (10,11) as an additional marker of inflammatory conditions. Nevertheless, it is noteworthy that the spatial distribution of the COX isoenzymes was
consistent with the localization of COX-1 (wound margin) and COX-2 (macrophages) in normal healing (22).

Observations from the present study support evidence that a reduction of PG levels is clearly associated with a diabetes-impaired chronic wound situation and, more importantly, that a COX-1–driven PG synthesis contributes to the outcome of the repair process. This hypothesis is consistent with the well-described pro- and anti-inflammatory properties of PGs (20). However, especially the COX-2 isoenzyme is generally implicated in the production of pro-inflammatory (21) and anti-inflammatory PGs. Selective inhibition of COX-2 activity in a model of carrageenan-induced pleurisy resulted in exacerbated late inflammatory conditions, which were not properly resolved (29). In this model, COX-2 was coupled with an increase in PGD₂ and 15-deoxy-Δ₁₂,14-PGJ₂, two PGs with prominent anti-inflammatory properties (29). In clear contrast to the above-mentioned animal models of inflammation, a COX-2–coupled PG synthesis does not significantly contribute to the inflammatory phase of skin repair. Selective inhibition of COX-2 during healing did not affect the outcome in different models of incisional and excisional skin repair (22,30,31). In accordance, observations from our present study of impaired healing strongly suggest that the biosynthesis of pro-inflammatory as well as anti-inflammatory PGs is most completely restricted to expression and activity of COX-1 in skin tissue. Remarkably, COX-1 has indeed been described to make an important contribution to inflammatory responses in a model of induced paw inflammation (32). This is in contrast to studies from animal models of induced gastric ulceration, which demonstrated a pivotal role for COX-2 in ulcer healing. Selective inhibition of COX-2 caused a severe disturbance of acute ulcer healing and proved to be fatal in the animals (33,34).

But, nevertheless, what are the reasons that might contribute to the restrictive “use” of a COX-1–coupled PG biosynthesis in healing skin tissue in contrast to other models of inflammation? Recent data have shown evidence for differential activities of COX-1 and -2 that depend on the availability of arachidonic acid (AA). Co-transfection experiments demonstrated that COX-1–coupled PG synthesis is favored over COX-2 in the presence of high concentrations of AA. Cytosolic phospholipase A₂ was especially able to drive PG synthesis from COX-1 via an increased supply of AA, which allowed a preferential coupling of COX-1 to membrane-associated PGE synthase 1 activities (35,36). For these reasons, one might hypothesize that basal levels of AA release differ markedly between different tissues and that these differences might be due to tissue-specific differences in phospholipase expression. Accordingly, two types of cytosolic phospholipase A₂ have been described for mouse epidermis in vivo (37), which might constitute levels of AA in skin tissue that are high enough to favor a COX-1–coupled over a COX-2–coupled PG biosynthetic pathway.

The role of the different COX-1–dependent PGs for skin repair might be diverse. PGE₂ strongly contributes to keratinocyte proliferation in vitro and in vivo. An initial study from Pentland and Needleman (38) demonstrated mitogenic effects of PGE₂ in cultured keratinocytes. In line with this, the mitogenic effect of the epidermal growth factor in murine keratinocytes could be attenuated by indomethacin (39). It has turned out that COX-1– and COX-2–deficient mice exhibited a strongly reduced tumorgenesis that was associated with a premature onset of keratinocyte terminal differentiation (40). For these reasons, it is tempting to speculate that reduced levels of PGE₂ in diabetes-impaired wound tissues might contribute to disturbed re-epithelialization in these animals. Moreover, PGD₂ and its metabolite 15-deoxy-Δ₁₂,14-PGJ₂ have been described as potent anti-inflammatory PGs (41,42). For these reasons, it has to be discussed that COX-1–derived PGs might also serve important anti-inflammatory actions in wound tissue that pivotally contribute to a tightly controlled resolution of inflammation. In summary, diabetes-impaired wound healing in ob/ob mice is characterized by reduced PGE₂/PGD₂ levels in the presence of a strongly elevated COX-2 expression at the wound site. This study suggests that a COX-1–coupled PG biosynthesis is associated with a normal wound repair process, but is strongly disturbed in an impaired wound repair process.

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REFERENCES


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COX IN DIABETIC WOUND REPAIR


