Bcl-2–Modifying Factor Induces Renal Proximal Tubular Cell Apoptosis in Diabetic Mice

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<td>doi:10.2337/db11-0141</td>
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This study investigated the mechanisms underlying tubular apoptosis in diabetes by identifying proapoptotic genes that are differentially upregulated by reactive oxygen species in renal proximal tubular cells (RPTCs) in models of diabetes. Total RNAs isolated from renal proximal tubules (RPTs) of 20-week-old heterozygous db/db, db/db, and db/db catalase (CAT)-transgenic (Tg) mice were used for DNA chip microarray analysis. Real-time quantitative PCR assays, immunohistochemistry, and mice rendered diabetic with streptozotocin were used to validate the proapoptotic gene expression in RPTs. Cultured rat RPTCs were used to confirm the apoptotic activity and regulation of proapoptotic gene expression. Additionally, studies in kidney tissues from patients with and without diabetes were used to confirm enhanced proapoptotic gene expression in RPTs. Bcl-2-modifying factor (Bmf) was differentially upregulated (P < 0.01) in RPTs of db/db mice compared with db/db and db/db CAT-Tg mice and in RPTs of streptozotocin-induced diabetic mice in which insulin reversed this finding. In vitro, Bmf cDNA overexpression in rat RPTCs coimmunoprecipitated with Bcl-2, enhanced caspase-3 activity, and Bmf induced apoptosis in RPTCs. More important, enhanced Bmf expression was detected in RPTs of kidneys from patients with diabetes. These data demonstrate differential upregulation of Bmf in diabetic RPTs and suggest a potential role for Bmf in regulating RPTC apoptosis and tubular atrophy in diabetes.

Although the classic view of diabetic nephropathy (DN) has focused on events leading to glomerular dysfunction, the gradual decline of renal function in later stages of DN is invariably associated with tubulointerstitial fibrosis and tubular atrophy (1). Indeed, tubulointerstitial fibrosis and tubular atrophy appear to be better predictors of late-stage renal disease progression than glomerular pathology (2–5). For example, examination of nephrons from proteinuric diabetic patients shows that 71% of glomeruli display glomerulotubular junction abnormalities and 8–17% of glomeruli are atubular glomeruli (6,7).

The mechanisms underlying tubular atrophy are incompletely delineated. Studies have shown that high glucose (HG) concentrations are associated with increased reactive oxygen species (ROS) production, which inhibits proximal tubular function and induces apoptosis (8–10). Apoptosis has been detected in renal proximal tubular cells (RPTCs) of diabetic mice (11,12) and rats (13,14) as well as in RPTCs of diabetic patients (15–17), suggesting that tubular apoptosis may precede tubular atrophy in atubular glomeruli. Although the link between ROS and tubular apoptosis seems clear, little is known about the genes involved in HG-induced RPTC apoptosis or ROS generation.

We previously reported that HG enhances angiotensinogen (Agt) gene expression via ROS generation in rat RPTCs in vitro (18,19) and that in vivo overexpression of rat Agt in RPTCs induces hypertension, albuminuria, and RPTC apoptosis in diabetes (20). Conversely, we also reported that RPTC-selective overexpression of catalase (CAT) attenuates ROS generation, tubulointerstitial fibrosis, and tubular apoptosis as well as proapoptotic gene expression in diabetic mouse kidneys in vivo (21,22). These data suggest that ROS generation may be directly or indirectly responsible for RPTC apoptosis in diabetes.

We now report that Bcl-2–modifying factor (Bmf), a proapoptotic gene that we identified via DNA chip microarray analysis, is differentially upregulated in RPTCs of db/db mice; we also validated this observation by immunohistochemistry and real-time quantitative PCR (qPCR). We further show enhanced Bmf expression in the RPTCs of mice with streptozotocin (STZ)-induced diabetes as well as in the kidneys of patients with diabetes. Finally, we found that Bmf overexpression enhances RPTC apoptosis and that HG in vitro induces Bmf mRNA expression via ROS generation and transforming growth factor-β1 (TGF-β1) expression.

RESEARCH DESIGN AND METHODS

Chemicals and constructs. D-glucose, D-mannitol, diphenylene iodium (DPI, an inhibitor of NADPH oxidase), rotenone (an inhibitor of mitochondrial electron transport chain complex I), apocynin (an inhibitor of NADPH oxidase), CAT, and monoclonal antibodies against β-actin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Normal glucose (5 mmol/L), Dulbecco’s Modified Eagle’s Medium (DMEM), 100× penicillin/streptomycin, FBS, and the expression vector pcDNA 3.1 were purchased from Invitrogen, Inc. (Burlington, ON, Canada). The caspase-3 activity assay kit was purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). Anti-Bmf and anti-c-Myc polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Active human TGF-β1 was purchased from R&D Systems (Hornby, ON, Canada). Scrambled Silent Negative Control #1 small interfering RNA (siRNA) and siRNAs for TGF-β1 and Bmf were procured from Qiagen, Inc.
CAT cDNA was a gift from Dr. Paul E. Epstein (University of Louisville, Louisville, KY). The plasmid pKAP2 containing the kidney-specific (Tg) mice (C57Bl/6 background) overexpressing rat CAT (rCAT) in transgenic mice overexpressing rat CAT (rCAT) in non-Tg, age- and sex-matched db/db littersmates served as controls. The physiologic parameters of db/db, +/+ db/db, and +/+ db/db CAT-Tg mice are displayed in Supplementary Table III. All animals received standard mouse chow and water ad libitum. Animal care and usage protocols were approved by the CHUM animal care committee.

**Mouse RTqPCR assays for gene expression.** Real-time qPCR assays for gene expression were performed on DN samples with a false discovery rate of 0.01. Preprocessed data were then subjected to filtering based on P < 0.05. The Gene Ontology database (32) for the different probe sets was used to detect the differentially regulated genes between the living donors and the DN samples with a false discovery rate of <0.1. The value “q” is a multiple testing corrected P value for false positives (briefly, the “q” value method first looks at the distribution of the P values, identifies at which value the P value becomes flattened, and then incorporates this information into the calculation of the adjusted P value).

**RESULTS**

**Microarray analysis.** Normalized data of the different probe sets by setting P < 0.05 on the chips generated a list of 21 upregulated and 25 downregulated genes associated with apoptosis in RPTCs of db/db mice compared with heterozygous db/m+ or db/db CAT-Tg mice (Supplementary Tables I and II). Our raw and normalized data are available in the public domain Gene Expression Omnibus National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19640).

The Gene Ontology database was used to screen for probe sets involved in apoptotic programs. Classification was further filtered by setting P < 0.01, compiling a list of five upregulated (Table 1) and six downregulated proapoptotic genes (Table 2) in db/db mice, compared with db/m+ and db/db CAT-Tg mice. We chose Bmf in the current study because its expression is at least threefold
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Proapoptotic genes upregulated in microarray chips of db/db vs. db/db+CAT-Tg mice overexpressing CAT

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<th>Fold-change</th>
<th>P</th>
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<tr>
<td>1452311_at</td>
<td>Baculoviral IAP repeat-containing 4</td>
<td>1.28</td>
<td>0.0039</td>
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<tr>
<td>1445452_at</td>
<td>TNF receptor-associated factor 1</td>
<td>1.99</td>
<td>0.0073</td>
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*The GO annotation package was used to select genes involved in the apoptosis process. The probe sets were selected with P < 0.01.

Proapoptotic genes downregulated in microarray chips of db/db vs. db/m+ and db/db vs. db/db+CAT-Tg mice overexpressing CAT

<table>
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<th>P</th>
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<tr>
<td>1417962_at</td>
<td>Growth hormone receptor</td>
<td>−2.92</td>
<td>0.0031</td>
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<tr>
<td>1419592_at</td>
<td>Unc-5 homolog C (C. elegans)</td>
<td>−1.53</td>
<td>0.0058</td>
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*The GO annotation package was used to select genes involved in the apoptosis process. The probe sets were selected with P < 0.01.
caspase-3 activity was determined in cell lysates. Expression of the fusion protein was confirmed by RT-PCR (Fig. 4A) and anti-Myc immunoblotting (Fig. 4B). Caspase-3 activity was significantly increased in lysates from cells transiently transfected with the Bmf fusion protein compared with lysates from cells transiently transfected with empty vector \((P < 0.005)\). Caspase-3 activity was further augmented...
when RPTCs were cultured in HG medium ($P < 0.005$; Fig. 4C). Knockdown of Bmf with siRNA reduced HG-induced apoptosis (TUNEL assay) in RPTCs compared with scrambled siRNA (Fig. 4D).

RPTCs transiently transfected with NH$_2$-terminally Myc-tagged Bmf exhibited fourfold increase in TUNEL-positive cells compared with empty vector-transfected cells ($P < 0.005$; Fig. 5A, and B). Parallel assays with lysates from transfected cells confirmed the expression of Myc-tagged Bmf (Fig. 5C).

**Bmf interacts with Bcl-2 in rat IRPTCs.** To investigate Bmf interaction with Bcl-2, coimmunoprecipitation experiments of Myc-Bmf with Bcl-2 were performed. Significant increases in coimmunoprecipitated Bcl-2 (Fig. 5D, a) but not Myc-Bmf (Fig. 5D, b) were observed in rat IRPTCs overexpressing Myc-Bmf cultured in HG medium.

**Bmf expression in diabetic human kidneys.** The clinical characteristics of the patients are shown in Supplementary Table VI. All had kidney cancer, which was the reason for the nephrectomies; some had T2DM, but others did not. Immunohistochemistry revealed Bmf expression in the renal distal tubules but not in the renal proximal tubules (RPTs) of the normal portions of human nephrectomy specimens from nondiabetic patients with kidney cancer (Fig. 6A, a–c). However, increased immunostaining for Bmf was observed in RPTs of the normal portions of nephrectomy specimens from patients with kidney cancer who also had diabetes (Fig. 6A, d–f). Interestingly, double immunostaining revealed frequent colocalization of Bmf overexpression in TUNEL-positive apoptotic RPTCs in diabetic kidney but not in nondiabetic kidney (Fig. 6B).

DNA gene chip microarray analysis (Affymetrix Gene Chip HGU 133plus 2 chip) of microdissected nephrons from seven patients with T2DM revealed modestly but significantly enhanced Bmf expression in glomeruli (1.48-fold increase) and tubulointerstitium (1.39-fold increase) compared with 18 control biopsy specimens from kidneys from living donors taken at the time of transplant ($q < 0.01$, where $q$ value is a multiple-testing corrected
FIG. 4. Overexpression of rat Bmf induces caspase-3 activity in rat RPTCs in vitro. A: RPTCs were transiently transfected by lipofectamine 2000 with the empty vector, pCMV-Myc or plasmid containing NH2-terminally tagged rat Bmf cDNA (pCMV-Myc rat Bmf). Expression of the Myc-rat Bmf mRNA was confirmed by conventional RT-PCR and quantified by densitometry. B: Anti-Myc immunoblotting was also performed. The nonspecific band serves as loading control. C: Caspase 3 activity in RPTCs transfected with empty vector (pCMV-Myc) or pCMV-Myc rat Bmf in normal glucose (□) and HG (■) media. Values are corrected to lysate protein levels. Values are the mean ± SEM, n = 4–8. ***P < 0.005. UV, ultraviolet. D: TUNEL images (a–d) and quantification of apoptotic cells (E) in vitro. After transfection with scrambled siRNA (a and c) or Bmf siRNA (b and d) in RPTCs cultured in normal glucose (NG, a and b) or HG (c and d) medium, cells were fixed and subjected to TUNEL (green) and DAPI (blue) staining (original magnification ×200). White arrows indicate TUNEL-positive cells; (e) quantification of TUNEL-positive cells (n = 3 experiments). **P < 0.01; N.S., not significant. (A high-quality color representation of this figure is available in the online issue.)
FIG. 5. Overexpression of rat Bmf increases TUNEL-positive cells and coimmunoprecipitates (Co-IP) with Bcl-2 in rat RPTCs in vitro. A: RPTCs were transiently transfected with the empty vector, pCMV-Myc, or pCMV-Myc rat Bmf. Cells were incubated for 24 h in 25 mmol/L d-glucose, then fixed and subjected to TUNEL and DAPI staining. TUNEL (green) and DAPI (blue) staining (original magnification ×200) are shown for RPTCs transfected with empty vector and pCMV-Myc rat Bmf. DNase-treated cells serve as TUNEL-positive controls. Cells left untreated with terminal transferase serve as a TUNEL-negative control. B: Quantification of TUNEL-positive cells per field is shown. Values are presented as percentages of TUNEL-positive cells/total cells per field ± SEM (n = 8 or 9). ***P < 0.005. C: Expression of the Myc-rat Bmf fusion protein was confirmed by anti-Myc immunoblotting, with β-actin as the loading control. D: Interaction of Myc-Bmf with Bcl-2 in rat RPTCs: (a) Immunoblotting for Bcl-2 in cytosolic fractions of rat RPTCs before (total lysate, TL) and after Co-IP with anti-Myc; (b) immunoblotting for Myc in cytosolic fractions of rat RPTCs on the same membrane after immunoblotting for anti-Bcl-2 in panel a. The relative density of Bcl-2 or Myc in RPTCs cultured normal glucose (5 mmol/L d-glucose plus 20 mmol/L d-mannitol DMEM) was expressed as controls (100 arbitrary units). Rabbit purified IgG (3 μg) was used as the control for Co-IP experiments. Values are the mean ± SEM for 4 independent experiments. ***P < 0.005; N.S., not significant. □, normal glucose; ■, high glucose medium. (A high-quality digital representation of this figure is available in the online issue.)
FIG. 6. Enhanced Bmf expression in RPTs from human kidneys from patients with diabetes. A: Bmf immunohistochemical staining in human kidney sections (original magnification ×200) from three nondiabetic cancer patients (a, patient with papillary variant carcinoma; b, patient with clear cell carcinoma; and c, patient with thyroid-like renal carcinoma) and three diabetic cancer patients (d, patient with papillary variant carcinoma and evidence of DN [nodular with tubular atrophy and interstitial fibrosis]; e, patient with clear cell carcinoma and no evidence of DN; and f, patient with clear cell carcinoma and nephroangiosclerosis with evidence of DN [secondary focal glomerulosclerosis, tubular atrophy, and interstitial fibrosis]). DT, distal tubule; G, glomerulus. B: Colocalization of Bmf expression and TUNEL-positive cells in human kidneys. Nondiabetic human kidney (patient I.D. #C) and diabetic human kidney with DN (patient I.D. #D) were sectioned, subjected to TUNEL assay to visualize apoptotic cells (green), and then incubated with anti-Bmf antibody, followed by anti-goat AlexaFluor 594 to demonstrate Bmf expression (red). Cells staining positively for TUNEL and Bmf appear yellow (merged image). Original magnification ×200. Arrows indicate cells that stained positively for TUNEL and Bmf. G, glomerulus; DT, distal tubule. (A high-quality color representation of this figure is available in the online issue.)
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*D* IABETES, VOL. 61, FEBRUARY 2012

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**DISCUSSION**

The current study documents enhanced Bmf expression in RPTCs of *db/db* mice, STZ-induced diabetic mice, and kidneys from patients with T2DM and shows that Bmf overexpression enhances RPTC apoptosis, indicating a potential role for Bmf in mediating tubular atrophy in the diabetic kidney.

To identify the proapoptotic genes regulated by ROS in *db/db* mice RPTCs, we used gene chip microarrays as an initial screen (37). A combination of high *P* values, fold changes, and Gene Ontology annotation (32) allowed us to obtain an overview of the genetic regulation of RPTC apoptosis occurring in murine models of T2DM.

We identified Bmf as one of five putative proapoptotic genes that were differentially upregulated (*P < 0.01 and >1.5-fold increase*) in RPTs of *db/db* mice compared with *db/+* and *db/db* CAT-Tg mice. The upregulation of Bmf mRNA and protein expression in RPTCs of *db/db* mice was confirmed by real-time qPCR and immunostaining.

To confirm enhanced Bmf expression, we used another model of diabetes, STZ-induced diabetes in mice (20). Our results document significant upregulation of Bmf expression in RPTCs of STZ-induced diabetes and its reversal by insulin.

There is evidence that HG induces apoptosis in RPTCs via ROS generation (20,38). Our present data show that HG also stimulates Bmf mRNA expression, which can be inhibited by rotenone, CAT, DPI, and apocynin. Cellular H$_2$O$_2$ and mitochondrial ROS levels were also significantly higher in RPTCs incubated in HG medium than in RPTCs cultured in normal glucose and were normalized in the presence of CAT (Supplementary Fig 2a and b). These observations indicate that ROS derived from mitochondrial oxidative metabolism may mediate, at least in part, HG-induced Bmf expression, which in turn would promote RPTC apoptosis.

To provide evidence that Bmf can directly induce RPTC apoptosis, we overexpressed Bmf in rat RPTCs. Transient transfection of RPTCs with rat Bmf cDNA resulted in activation of caspase-3 parallel with increases in the number of apoptotic cells, similar to that observed in transfected fibroblasts and cancer cells (39). We were unable to detect increases in endogenous rat Bmf expression in RPTCs by immunoblotting or immunofluorescence (data not shown) using the same anti-Bmf antibodies used for immunohistochemistry. Of note, Schmelzle et al. (40) also observed that commercially available anti-Bmf antibodies do not work in immunoblotting versus immunostaining of Bmf.

The precise mechanism(s) by which HG evokes Bmf expression in RPTC apoptosis remains unclear. Our present data show that TGF-β1 stimulates Bmf mRNA expression and that knockdown of TGF-β1 with siRNA attenuates Bmf mRNA expression in RPTCs in HG. These observations are consistent with findings of Ranjaun et al. (41) showing that TGF-β1 stimulates Bmf gene expression. ROS could induce phosphorylation of Bmf via c-Jun NH$_2$-terminal kinase (42) or other signaling pathways. Phosphorylated Bmf would then translocate from the cytoskeleton to bind proapoptotic proteins (i.e., Bcl-2 and Bcl-xL), which are located on the mitochondria, to interfere with Bcl-2/Bcl-xL mitochondrial gatekeeping and thus allow enhanced Bax binding to the mitochondrion. This would result in the collapse of the mitochondrial transmembrane potential and thus activation of the intrinsic pathway of apoptosis. Our findings that Myc-Bmf coimmunoprecipitates Bcl-2 in rat RPTCs lend further support to this notion.

We searched the publicly accessible National Center for Biotechnology Information data bank (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1009) for microarray studies performed on biopsy specimens from patients with DN (Supplementary Table V) to confirm our findings in *db/db* mice. Bmf was not detectable, however, in human kidneys from patients with diabetes when the first generation of chips (Affymetrix Human Genome U95 Version 2 Array) was used because a Bmf probe set was not present in these chips. Bmf expression was upregulated, however, in kidney allografts from living donors compared with at implantation before revascularization (0 months) using the Affymetrix Human Genome U133 Plus 2.0 Array (Affy 133plus chip), which contains the Bmf probe set. These findings indicate that Bmf expression may be upregulated in human kidneys under pathologic conditions, including DN.

Indeed, this possibility is supported by our immunohistochemistry studies that show increased Bmf expression in RPTs taken from nonmalignant portions of kidneys removed from diabetic patients due to kidney cancer compared with those taken from nondiabetic patients with kidney cancer. Interestingly, RPTCs overexpressing Bmf frequently stained positive for TUNEL in diabetic human kidney. Furthermore, the Affy 133plus chip assay revealed significantly upregulated Bmf expression (q < 0.01, where q value is a multiple testing corrected *P* value) in microdissected glomeruli (1.48-fold increase) and tubulo-interstitium (1.39-fold increase) from patients with T2DM compared with nondiabetic patients (kidney donors). The 1.39-fold versus 3-fold increases in Bmf expression in human diabetic tubulo-interstitium and *db/db* mouse RPTs, respectively, are difficult to reconcile. It is possible that Bmf mRNA expression in human tubulo-interstitium might have been underestimated because of the use of a mixture of tubules and interstitium versus the >90% purity of our *db/db* mouse RPT fraction. Additional qPCR studies are needed to compare Bmf expression in human RPTs with or without T2DM.

Our results may have clinical implications for patients with T2DM. Because tubular apoptosis is one of the characteristic morphologic changes in human diabetic kidneys (15–17) and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology (2–4), we suggest that RPTC apoptosis may be an initial mechanism for tubular atrophy in T2DM. Our present data point toward Bmf as one of the mediators of this process. However, whether enhanced Bmf expression directly or indirectly induces RPTC apoptosis in human T2DM remains to be investigated.

In summary, the current study suggests an important role for Bmf in mediating RPTC apoptosis in the diabetic mouse kidney in vivo, and, likely, in diabetic human kidneys. Our observations raise the possibility that selective targeting of this proapoptotic protein may provide a novel approach in preventing or reversing the pathologic manifestations of DN, particularly tubular atrophy.

**ACKNOWLEDGMENTS**

This work was supported, in part, by grants from the Canadian Institutes of Health Research (MOP 84363 and MOP 106688) to J.S.D.C., the Kidney Foundation of Canada
The authors thank all participating centers of the European Renal cDNA Bank-Kroener-Fresenius biopsy bank (ERCB-KFB) and their patients for their cooperation. Active members at the time of the study: Clemens David Bank (ERCB-KFB) and their patients for their cooperation. European Renal cDNA Bank-Kroener-Fresenius biopsy bank (ERCB-KFB) and their patients for their cooperation. No potential conflicts of interest relevant to this article were reported.

G.J.L. and N.G. researched data, contributed to discussion, and wrote the manuscript. H.M., C.-S.L., J.X.Z., and J.S.D.C. contributed to discussion and reviewed and edited manuscript. J.S.D.C. contributed to discussion and wrote, reviewed, and edited the manuscript.

The authors thank all participating centers of the European Renal cDNA Bank-Kroener-Fresenius biopsy bank (ERCB-KFB) and their patients for their cooperation. Active members at the time of the study: Clemens David Bank (ERCB-KFB) and their patients for their cooperation.

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