Activation of Intrinsic and Extrinsic Proapoptotic Signaling Pathways in Interleukin-18-mediated Human Cardiac Endothelial Cell Death*

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Endothelial cells are the primary targets of circulating immune and inflammatory mediators. We hypothesize that interleukin-18, a proinflammatory cytokine, induces endothelial cell apoptosis. Human cardiac microvascular endothelial cells (HCMEC) were treated with interleukin (IL) 18, mRNA expression was analyzed by ribonuclease protection assay, protein levels by immunoblotting, and cell death by enzyme-linked immunosorbent assay and fluorescence-activated cell sorter analysis. We also investigated the signal transduction pathways involved in IL-18-mediated cell death. Treatment of HCMEC with IL-18 increases 1) NF-kB DNA binding activity; 2) induces kB-driven luciferase activity; 3) induces IL-1β and TNF-α expression via NF-kB activation; 4) inhibits anti-apoptotic Bcl-2 and Bcl-XL; 5) up-regulates proapoptotic Fas, Fas-L, and Bcl-Xs expression; 6) induces fas and Fas-L promoter activities via NF-kB activation; 7) activates caspases-3, -9, -9, and BID; 8) induces cytochrome c release into the cytoplasm; 9) inhibits FLIP; and 10) induces HCMEC cell death by apoptosis as seen by increased annexin V staining and increased levels of mono- and oligonucleosomal fragmented DNA. Whereas overexpression of Bcl-2 significantly attenuated IL-18-induced endothelial cell apoptosis, Bcl-2/Bcl-xL chimeric phosphorothioate 2′-MOE-modified antisense oligonucleotides potentiated the proapoptotic effects of IL-18. Furthermore, caspase-8, IKK-α, and NF-κB p65 knockdown or dominant-negative IκB-α and dominant-negative IκB-β or kinase dead IκB-κ significantly attenuated IL-18-induced HCMEC cell death. Effects of IL-18 on cell death are direct and are not mediated by intermediaries such as IL-1β, tumor necrosis factor-α, or interferon-γ. Taken together, our results indicate that IL-18 activates both intrinsic and extrinsic proapoptotic signaling pathways, induces endothelial cell death, and thereby may play a role in myocardial inflammation and injury.

Under physiological conditions, apoptosis or programmed cell death plays a vital role in endothelial and smooth muscle cell homeostasis. However, apoptosis of endothelial cells plays a pathological role during ischemia, ischemia/reperfusion injury, infarction, atherogenesis, and atherosclerosis. Interactions between endothelial cells and immune cells during immune and inflammatory disorders and generation of abnormally high levels of reactive oxygen and nitrogen intermediates have also been shown to induce endothelial cell injury (1–5). In addition, several proinflammatory cytokines negatively regulate endothelial cell survival (4, 5). However, the role of interleukin (IL)-18, a proinflammatory cytokine, known previously as an IFN-γ inducing factor (6, 7), in cardiac endothelial cell apoptosis is not known.

IL-18 belongs to the IL-1 family, and has similar biological functions as that of IL-1β (6, 7). Both IL-1β and IL-18 are synthesized as proforms, and are cleaved by IL-1β converting enzyme (caspase-1) to a mature, biologically active, and secreted form (8). IL-18 is induced during various immune, infectious, and inflammatory disorders, and further amplifies the inflammatory cascade by inducing the expression of other proinflammatory cytokines and adhesion molecules (6, 7). It induces the expression of several ELR+ CXC chemokines, and attracts and activates polymorphonuclear leukocytes to the site of injury or inflammation (6, 7, 9, 10). Recently, IL-18 has also been shown to be a potent chemoattractant for CD4+ T cells both in vivo and in vitro (11), implicating its proinflammatory role in immune and autoimmune disorders.

IL-18 signals via the IL-18 receptor. IL-18R comprises α and β subunits (12, 13). Whereas IL-18Rα acts as a ligand binding subunit, the β subunit transduces intracellular signals. Binding of IL-18 to its receptors results in the recruitment of

* This work was supported in part by American Heart Association, National Center Grant-in-aid 0150105N and the National Institutes of Health NHLBI Grant HL68020. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† Supported by a grant from the American Heart Association, Texas Affiliate, and the Merit Review Entry Program of the Department of Veterans Affairs.
MyD88, an adaptor molecule also involved in IL-1 and Toll-like receptor signaling, followed by IRAK and TRAF6 (14, 15). IL-18 also signals via c-Jun N-terminal kinase (14) leading to NF-κB activation (16, 17). Activation of NF-κB results in the induction of several inflammatory mediators including IL-18 and IL-18-mediated NF-κB-dependent gene transcription (6, 7, 10, 18). For example, in rheumatoid arthritis synovial fibroblasts, Merel et al. (10) have demonstrated that IL-18 activates both NF-κB and NF-κB-dependent IL-8, epithelial neutrophil-activating peptide 78, and Gro-α (26).

In addition to its stimulatory effects on cytokines, chemokines, and adhesion molecules, IL-18, in combination with IL-2, has been shown to induce tumor cell death via the Fas-Fas-L pathway (20), and in combination with IL-12, induced natural killer cell-mediated tumor cell death (21). Furthermore, concurrent administration of IL-18 and IL-12 induced larcimal and salivary gland atrophy with epithelial cell apoptosis (22). However, IL-18, by itself, has not been shown to induce cell death. In addition, the signal transduction pathways elicited by IL-18 in endothelial cell death have yet to be explored. In the present study, we demonstrate that IL-18 activates NF-κB, induces IL-1β and TNF-α expression, up-regulates the proapoptotic Fas, Fas-L, and Bcl-Xs expression, down-regulates the antiapoptotic Bcl-2 and Bcl-Xl gene expression, activates caspases-8, -3, and -9, and inhibits the long isoform of FLIP, and induces HCMEC death independent of IL-1β, TNF-α, and IFN-γ.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human cardiac microvascular endothelial cells (HCMEC) were obtained from ScienCell Research Laboratories (San Diego, CA). They were characterized by the immunofluorescent method using antibodies directed against VWF/Factor VIII and CD31 (P-CAM) and by uptake of DiI-Ac-LDL. The cells were grown in endothelial cell medium supplied by the manufacturer and supplemented with 5% serum (complete media). At 70–80% confluency, the complete media was replaced with media containing 0.5% BSA. After overnight incubation, recombinant human (rh) IL-18 (R&D Systems) was added and cultured for the indicated time periods. At the end of the experiment, culture supernatants were collected and snap frozen. Cells were harvested, snap frozen, and stored at −80 °C. To determine whether IL-18 induces NF-κB activation directly or is mediated by intermediaries such as IL-1β, TNF-α, or IFN-γ, cells were pretreated with the respective neutralizing antibodies (goat-anti-human IL-1β (AB-201-NA), TNF-α (AF-210-NA), IFN-γ (AF-285-NA) antibodies; R&D Systems; 5 μg/ml for 1 h) prior to IL-18 addition. Normal goat IgG (AB-108-C; R&D Systems) served as control. Efficacy of these antibodies was verified in transfection assays using HCMEC transiently transfected with pN-B-Luc vector that contains five copies of the NF-κB consensus sequence linked to the minimal E1B promoter-luciferase reporter gene (23) or pG5-Luc vector that contains four copies of GAS consensus sequence linked to the minimal E1B promoter-reporter gene (23). Transfection efficiency by dividing firefly luciferase activity with that of an internal control. Cells extract were prepared, and luciferase activities were determined with a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) using the Promega Bioluminescent dual luciferase reporter assay system (22). Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of corresponding Renilla luciferase, and expressed as mean relative stimulation ± S.E. for a representative experiment from three separate experiments, each performed in triplicate. After transfection cells were found viable as seen by trypan blue dye exclusion. Cells were also treated with phosphorothiolated TNF-α antiserum (5-CAGTGCCTGATTGCTGTC3′) or scrambled (5-CGATGCTCTGGGTTTCC-3′) oligonucleotides (24) using OligofectAMINE (Invitrogen, Carlsbad, CA) prior to IL-18 treatment. Efficacy of TNF-α antiserum oligonucleotides was verified in lipopolysaccharide-treated (1 μg/ml for 24 h; Escherichia coli 055:B5, Sigma) HCMEC.

To determine the signal transduction pathways involved in IL-18-mediated NF-κB-dependent cytokine induction, cells were transiently transfected with dnIRAK in pCI-Neo (25), dnTRAF-6 (pPRK5-TRAF6 (289–522); Flag, 25) or transfected with 5 nmol of double stranded MyD88 small interfering RNA (sense: 5′-CUGCAAGACACAAAUUCUdTdT-3′ (26)) TAK1 siRNA (sense 5′-UGCUGUACUCUAACUCCGGA-3′ (27)), and p56 siRNA (sense, 5′-UGACGUGAAGGUCUCCUGGGGAGdTdT (28)) using OligofectAMINE, luciferase activity was analyzed (23). Double stranded scrambled siRNA were used as a control (scrambled siRNA; 5′-UCUGCGCUUUCACUGUGGdtdTdT). Knockdown of respective proteins were confirmed after 72 h post-transfection by Western blotting. NF-κB inhibition was also achieved by transfecting cells with IKK-α siRNA expression vector (IMGENEX Corp., San Diego, CA), or transiently transfected with dnIk-κBα (pdDNA3-1KK-α-HA), dnIκB-α (pCMX-IκB-α-S32A/S36A), or dnIκB-β (pCMV-Tag3B-IκB-β (S19A/S32A)-Myc) using Lipofectamine reagent (23). Empty vectors were used as controls. Inhibition of caspases-1 and -8 was achieved by transfecting HCMEC with caspase-1 and caspase-8 siRNA expression vectors (IMGENEX), or pretreating (30 min) cells with Z-DEVD-FMK (a caspase-3 inhibitor; 50 μM), Z-LEHD-FMK (a caspase-9 inhibitor; 20 μM), Z-VAD-FMK (a pan-caspase inhibitor, 50 μM), or Z-FA-FMK (a negative control; 50 μM) in MeSO or MeSO alone. Caspase inhibitors were obtained from Enzyme Systems Products (Livermore, CA).

**Electrophoretic Mobility Shift Assay**—NF-κB DNA binding activities were measured by electrophoretic mobility shift assay (EMSA). Preparation of nuclear protein extracts and EMSA were performed as described earlier (24). The following double-stranded oligonucleotides were used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA): NF-κB, 5′-AGTTGAGGGGACTTCCAGGC-3′; mutant, 5′-AGTTGAGGGGACTTCCAGGC-3′; and NF-κB binding site (pLuc-4X-530) or IL-18 promoter (pLuc-MCS) containing nucleotides −308 to −19 that contains a B-like motif was also analyzed (28). The Fas-L promoter (proximal: sense; 5′-AGACAGAGGTTGTTTCTCTTACGTAGA-3′; between −62 and −34 nt; distal: sense; 5′-GGCTCTAGTTCGTCCTCCTCAGAATT-3′; and nucleotides between −543 and −514 (29)) were also used. In the absence of protein extract, competition with 100-fold molar excess unlabeled or corresponding mutant oligonucleotides served as controls. Gel supershift assays were performed as previously described (23) using anti-p50 (C-19; sc-1190 X) and anti-p65 (C-20; sc-372 X) antibodies (Santa Cruz Biotechnology, Inc.).

**fas and Fas-L Promoter Analyses**—HCMEC were transiently transfected with the promoterless fas promoter-reporter vector (pLuc-MCS) containing fas L nucleotides from −1379 to −19 in either sense or antisense (−19 to −1739) orientations (28). Owen-Schua and colleagues (28) have previously demonstrated that fas promoter is orientation-dependent and when present in an antisense orientation it is inactive both at basal and stimulated conditions. Therefore, the antisense (−19 to −1739) construct served as an additional control. In addition, the fas nucleotide sequence −308 to −19 that contains a B-like motif was also analyzed (28). The Fas-L (CD95L) promoter-reporter construct (−860/+100) in pTATA-Luc was transiently transfected with Lipofectamine reagent as described previously (29). Corresponding empty vectors also served as controls. Cells were cotransfected with the dnIκB-α expression vector. In addition, cells were cotransfected with pCMX-IκB-α-S32A/S36A with 50 μg of pCR-Luc (S32A/S36A) for 24 h and Fas-L promoter (proximal: sense; 5′-GACACAGGGGCTTTTGTGACTATGAA-3′; between −62 and −34 nt; distal: sense; 5′-GGCTCTAGTTCGTCCTCCTCCTCAGAATT-3′; and nucleotides between −543 and −514 (29)) were also used. In the absence of protein extract, competition with 100-fold molar excess unlabeled or corresponding mutant oligonucleotides served as controls. Gel supershift assays were performed as previously described (23) using anti-p50 (C-19; sc-1190 X) and anti-p65 (C-20; sc-372 X) antibodies (Santa Cruz Biotechnology, Inc.).

To confirm our EMSA results, we have also analyzed B-driven luciferase activity using the pN-B-Luc vector using Lipofectamine reagent. pEGFP-Luc served as a control. The pRL Renilla luciferase reporter gene (100 ng; pRL-TK vector, Promega) was used as an internal control. Luciferase activities were determined as previously described (23). Transfection efficiency of HCMEC was determined as previously described using pEGFP-N1 vector (Clontech) that constitutively expresses EGFP under regulation of the cytomegalovirus pro-
IL-18 Induces Endothelial Cell Death

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Cell Death—After 48 h incubation in endothelial cell medium + 0.5% BSA, HCEMC were treated with IL-18 (100 ng/ml) for up to 24 h. Floating cells were collected and added to the scraped adherent cells, and analyzed for apoptosis using the annexin V-PI/C detection kit (Oncogene Research Products, San Diego, CA) that detects phosphatidyserine on the outer surface of the cell membrane. Cells were counterstained with propidium iodide and analyzed by flow cytometry. Apoptosis was also analyzed by quantitating mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates by an ELISA (Cell Death ELISA kit; Roche Diagnostics) (33). After 48 h incubation in endothelial cell medium + 0.5% BSA, cells were treated with IL-18 (100 ng/ml) for 24 h, harvested, cytoplasmatic fractions were extracted, and analyzed for mono- and oligonucleosomes. IL-18-mediated cell death was also analyzed by quantitating cytochrome c release. Mitochondrial and cytoplasmatic fractions were prepared using the Mitochondria Isolation Kit (Biovision, Milpitas, CA). Cytochrome c levels were measured colorimetrically using a commercially available kit (FunctionELISA Cytochrome c kit, Active Motif).

Statistical Analysis—Comparisons between experimental groups were made using the unpaired t test with Bonferroni correction for multiple comparisons, if needed. If three comparisons were made, a p value of <0.025 was considered significant. For two comparisons, a p value of <0.05 was considered significant. Each experiment was performed at least three times and group data were expressed as mean ± S.E.

RESULTS

IL-18 Increases NF-κB DNA Binding Activity and Induces κB-driven Luciferase Activity in HCEMC—IL-18 exerts its biological effects upon binding to its cognate receptor (IL-18R), a heterodimer comprised of a ligand-binding subunit IL-18Rα and the signal transducing subunit IL-18Rβ. Northern blot analysis using 2 μg of poly(A)+ RNA revealed expression of both IL-18Rα and -β in HCEMC at basal conditions (Fig. 1A, left panels). Furthermore, Western blot analysis using 40 μg of membrane extracts revealed IL-18Rα and -β expression (Fig. 1A, right panels), indicating that HCEMC express both mRNA and protein for 18Rα and -β at basal conditions. IL-18 has previously been shown to activate NF-κB in various immune and non-immune cells (6, 7). Because activation of NF-κB plays a pivotal role in the regulation of pro- and anti-apoptotic gene expression (18), in the next series of experiments, we investigated the effects of IL-18 on NF-κB DNA binding activity and κB-driven luciferase activity in HCEMC. Fig. 1B shows that HCEMC express low levels of NF-κB DNA binding activity at basal conditions. In contrast, treatment with IL-18 for 1 h increased NF-κB levels in a dose-dependent manner with peak levels detected at 100 ng/ml. No further increase in NF-κB activity was detected when IL-18 levels were further increased to 500 ng/ml. Therefore, in all subsequent experiments, we used IL-18 at 100 ng/ml. Time course studies revealed a slight increase in NF-κB activation at 30 min, increased further at 1 h, and remained at these high levels up to the 3-h study period (Fig. 1C). To further confirm the stimulatory effects of IL-18 on NF-κB activation, HCEMC transiently transfected with κB-driven luciferase vector were treated with IL-18. Our results indicate that IL-18 significantly increases κB-driven luciferase activity (7.2-fold; p < 0.001), but not EGF-driven, luciferase activity (Fig. 1D), and preincubation with IL-1B, TNF-α, or IFN-γ neutralizing antibodies failed to modulate IL-18-mediated κB-driven luciferase activity, indicating that IL-18 is a potent and direct inducer of NF-κB activation. However, the anti-IL-1B neutralizing antibodies significantly inhibited IL-1B-mediated κB-driven luciferase activity (Fig. 1E, left panel). Similarly, the anti-TNF-α neutralizing antibodies significantly inhibited TNF-α-mediated κB-driven luciferase activity (Fig. 1E, middle panel). The anti-IFN-γ antibodies inhibited IFN-γ-mediated GAS-driven luciferase activity (Fig. 1E, right panel), indicating that these antibodies neutralize the biological activities of their respective recombinant proteins. Gel supershift assays revealed a supershift when nuclear protein extracts were preincubated with either anti-p50 or -p65 antibodies, indicating that...
FIG. 1. IL-18 increases NF-κB DNA binding activity and κB-driven luciferase activity in HCMEC. HCMEC were grown in endothelial cell medium supplemented with 5% serum. At 70% confluency, the complete medium was replaced with medium containing 0.5% BSA. After overnight culture, total RNA was isolated, enriched for poly(A) RNA, and expression of IL-18Rα and -β was analyzed by Northern blot analysis using 2 μg of poly(A) RNA and 32P-labeled cDNA (A, upper panels). IL-18Rα and -β protein levels were analyzed by Western blot analysis using 30 μg of membrane extracts and subunit-specific antibodies (A, lower panels). To determine the effects of IL-18 on NF-κB DNA binding activity, HCMEC were treated with various concentrations of rhIL-18 for 1 h. Cells were harvested and nuclear proteins were extracted and analyzed for NF-κB DNA binding activity by EMSA (B). Time course studies were performed using 100 ng/ml IL-18 (C). To further confirm our EMSA results, we also performed transient transfection assays using a NF-κB promoter-reporter construct (pNF-κB-Luc). Cells were co-transfected with pRL-TK vector. pEGFP-Luc was used as a control. 24 h after transfection, HCMEC were treated with IL-18 (100 ng/ml), and analyzed for Renilla and firefly luciferase activities (D). HCMEC were pretreated for 1 h with anti-IL-1, TNF-α, or IFN-γ neutralizing antibodies (5 mg/ml) prior to IL-18 addition. Normal IgG (preimmune) served as isotype control. κB-dependent luciferase activity was determined 7 h post-IL-18 treatment. *, p < 0.001 versus IL-18-treated pEGFP-Luc-transfected cells. Efficacy of IL-18, TNF-α, and IFN-γ antibodies was determined in transient transfection assays. HCMEC were transfected with either pNF-κB-Luc or pGAS-Luc vectors. pEGFP-Luc served as a control. Cells were co-transfected with pRL Renilla luciferase (pRL-TK vector) vector. 24 h after transfection, cells were treated with neutralizing antibodies (5 μg/ml) for 1 h prior to the addition of the respective recombinant protein. 7 h later, luciferase activities were determined (E). *, p < 0.01 versus untreated and respective pEGFP-Luc-transfected cells; †, p < 0.01 versus respective cytokine-treated pNF-κB-Luc (IL-18, TNF-α) or pGAS-Luc (IFN-γ)-transfected cells. Gel supershift assays were performed using p50 and p65-specific antibodies (F). Lanes 1–3 in panels B, C, and F; lane 1, competition with mutant NF-κB oligonucleotide. Protein extract from HCMEC treated with IL-18 for 1 h was preincubated with a 50-fold molar excess of unlabeled double-stranded mutant NF-κB oligonucleotide followed by the addition of 32P-labeled consensus κB probe. Lane 2, competition with consensus NF-κB oligonucleotide. Protein extract from HCMEC treated with IL-18 for 1 h was preincubated with 50-fold molar excess of unlabeled double-stranded consensus NF-κB oligonucleotide followed by the addition of 32P-labeled consensus κB probe. Lane 3, no protein extract, but contains 32P-labeled consensus κB probe. Arrows indicate κB-specific DNA-protein complexes. Unincorporated labeled probe that runs to the bottom of the gels is not shown in these panels.
IL-18 Induces IL-1β and TNF-α Expression via NF-κB Activation—IL-18 is a potent inducer of NF-κB activation (Fig. 1). Because IL-1β and TNF-α are κB-responsive proinflammatory cytokines and play an important role in myocardial inflammation and injury (34, 35), we then explored the effects of IL-18 on IL-1β and TNF-α expression and determined whether IL-18 induces cytokine expression via NF-κB activation. Fig. 2A shows that both IL-1β and TNF-α are expressed at low levels in HCMEC at basal conditions, and treatment with IL-18 significantly increased their expression (densitometry, ratio of specific gene to that of their corresponding L32 expression; IL-1β, 5.13-fold, \( p < 0.01 \); TNF-α, 4.98-fold, \( p < 0.001 \)). Similar to their mRNA expression, IL-18 also increased IL-1β and TNF-α protein levels (Fig. 2B; IL-1β, 4.52-fold; TNF-α, 4.38-fold, both \( p < 0.01 \)) and increased their secretion into culture supernatants (Fig. 2C, \( p < 0.005 \) versus control). Furthermore, knockdown of p65 by p65 siRNA or overexpression of dnIkB-α attenuated IL-18-mediated NF-κB (Fig. 2D), but not Oct-1 (Fig. 2E) levels. In addition, IL-1β and TNF-α mRNA expression was also attenuated (Fig. 2F). We further explored possible signal transduction pathways involved in IL-18-mediated cytokine expression using pathway-specific dominant negative and kinase dead expression vectors, and siRNA. Knockdown of TAK1, IKK-β, and p65 following siRNA transfection was confirmed by Western blotting (Fig. 2G). Transfection with diRNAK, dnTRAF6, dnIKK-γ, dnIkB-α, dnIkB- β, kdIKK-β, and knockdown of MyD88, TAK1, and p65 significantly inhibited IL-18-mediated IL-1β and TNF-α secretion in HCMEC (Fig. 2H). However, transfections with empty vectors or scrambled siRNA (IMG-800–6) failed to modulate IL-18-mediated cytokine secretion (Fig. 2H), indicating that IL-18 is a proinflammatory cytokine and signals via MyD88-IRAK-TRAF6-IKK-IκB.

IL-18 Induces Pro-apoptotic Fas, Fas-L, and Bcl-\( X_\alpha \) Expression and Inhibits Anti-apoptotic Bcl-2 and Bcl-\( X_\beta \) Expression—To determine whether IL-18 regulates apoptotic gene expression, we analyzed HCMEC for pro- and anti-apoptotic gene expression using RPA (Fig. 3). Our results indicate that while IL-18 up-regulated Fas expression and induced Fas-L and Bcl-\( X_\alpha \) expression, it significantly inhibited anti-apoptotic Bcl-2 mRNA levels (Fig. 3, A–C). Western blot analysis revealed significantly low Bcl-\( X_\alpha \) with concomitant induction of anti-apoptotic Bcl-\( X_\beta \) protein levels (Fig. 2, D and E). Because IL-18 increased Fas and Fas-L expression, we then studied whether IL-18 regulates their promoter activities. HCMEC were transiently transfected with the \( f as \) promoter-reporter construct containing the sequence from -1739 to -19 nucleotides upstream of \( f as \) ATG (28). Our results indicate that treatment with IL-18 increases \( f as \) promoter-driven luciferase activity by at least 4-fold (\( p < \) versus corresponding control; Fig. 4A). However, IL-18 failed to induce \( f as \) promoter activity when the \( f as \) promoter sequence was cloned in an antisense orientation (–19/–1739 nt). In addition, IL-18 significantly increased \( f as \) promoter-driven luciferase activity from a deletion construct containing an NF-κB binding site (–306/–19 nt) (28). Co-transfection with diIkB-α significantly attenuated IL-18-mediated \( f as \) promoter activity (Fig. 4A). To further confirm our results, HCMEC were transfected with pLuc-Fas that contained 3 copies of the NF-κB site from the \( f as \) promoter (28). Whereas transfection with the empty vector (pLuc-MCS) had minimal effects on luciferase activity, treatment with IL-18 significantly increased κB-driven luciferase activity that was attenuated by the overexpression phosphorylation deficient nkB-α (Fig. 4B). Similarly, IL-18 increased Fas-L promoter activity in a NF-κB-dependent manner (Fig. 4C). Furthermore, transfection with reporter constructs containing multimers of either proximal or distal κB binding sites from Fas-L (29) indicated that IL-18 is a potent activator of NF-κB (Fig. 4D), and induces \( f as \) and Fas-L promoter activities via NF-κB activation. To further confirm our promoter-reporter studies (Fig. 4, B–D), we also performed EMSA using double stranded oligonucleotides containing NF-κB binding sites from \( f as \) (28) and Fas-L (29) promoters. Results shown in Fig. 4E demonstrate that, indeed, IL-18 increases NF-κB DNA binding activity in HCMEC.

IL-18 Induces HCMEC Death—Because IL-18 induced proinflammatory and proapoptotic gene expression, we hypothesized that IL-18 will induce HCMEC death. Therefore, we analyzed HCMEC death by two independent methods: annexin V staining that detects the translocated phosphatidylserine in the outer membrane and indicates early stages of apoptosis, and the measurement of mono- and oligonucleosomal fragmented DNA by ELISA in the cytoplasmatic extracts. Our results indicate that treatment with IL-18 increased annexin V positive cells at 8 h, increased further at 12 h, and peaked at 24 h with nearly 40% of cells positive for annexin V (Fig. 5A). Preincubation with anti-IL-18 neutralizing antibodies, but not control IgG, significantly attenuated IL-18-induced cell death (\( p < 0.001 \); Fig. 5A). ELISA of mono- and oligonucleosomal fragmented DNA further confirmed our results obtained with annexin V staining (Fig. 5B). Preincubation with anti-IL-18 neutralizing antibodies, but not IL-1β, TNF-α, or IFN-γ neutralizing antibodies, or control IgG, attenuated IL-18-induced cell death. Neutralization of IL-1β and TNF-α together also failed to modulate IL-18-induced endothelial cell death (data not shown). Furthermore, knockdown of caspase-1, a cysteinyl protease that cleaves pro-IL-1β and pro-IL-18 to their mature forms (36) (knockdown of caspase-1 protein was confirmed by Western blotting, Fig. 5C), as well as treatment with TNF-α antisense oligonucleotides that inhibit TNF-α protein synthesis failed to modulate IL-18-induced cell death (Fig. 5B). Efficacy of TNF-α antisense oligonucleotides was confirmed in studies in which lipopolysaccharide-mediated TNF-α secretion was significantly attenuated by TNF-α antisense, but not the scrambled, oligonucleotides (Fig. 5D). In contrast, transfection with diIkB-α, diIkB-β, kdIKK-β, or knockdown of p65 or IKK-α significantly attenuated IL-18-induced cell death (Fig. 5E). Because IL-18 attenuated Bcl-2 expression (Fig. 3A), we hypothesized that overexpression of Bcl-2 will overcome the proapoptotic effects of IL-18. Indeed, overexpression of Bcl-2 significantly attenuated IL-18-induced cell death (Fig. 5F). On the other hand, inhibition of Bcl-2/Bcl-\( X_\alpha \) using bicspecific antisense oligonucleotides potentiated IL-18-mediated cell death. These results indicate that IL-18 is a proapoptotic cytokine and induces HCMEC death via activation of NF-κB.

IL-18 Induces HCMEC Death via Activation of Caspases-8 and -3—Activation of both upstream (e.g. caspase-8) and effector (e.g. caspase-3) caspases play a central role in the execution of apoptosis. Therefore, we investigated the role of caspases-8 and -3 in IL-18-mediated cell death. Western blot analysis using caspase-8-specific antibodies that detect both the full-length procaspase-8 and the cleaved active forms revealed that treatment with IL-18 activates caspase-8 as seen by the formation of increased levels of cleaved products (p43/p41 and p18; Fig. 6A). FLIP, an inhibitor of Fas-mediated death signaling (37), is expressed at high levels in the endothelial cells, and treatment with IL-18 significantly attenuated (\( p < 0.01 \) versus untreated) its expression (Fig. 6B). Treatment with IL-18 also induced caspase-3 activation (Fig. 6C). Pretreatment with the cell permeable caspase-8 inhibitor (Z-
IL-18 induces iκB-dependent IL-1β and TNF-α expression in human cardiac microvascular endothelial cells. A, HCMEC at 80% confluency were treated with rhIL-18 (100 ng/ml) for 2 h. 20 μg of total RNA was isolated and analyzed for IL-1β and TNF-α expression by RPA. Large ribosomal RNA (L32) was used as an internal control. Numbers at the top denote RNA isolated from three independent experiments. B, IL-1β and TNF-α protein levels were determined by Western blot analysis using 30 μg of protein extract. β-Actin was used as an internal control. IL-1β and TNF-α levels in culture supernatants at 2 h post-IL-18 treatment were determined by ELISA (C). *, p < 0.005 versus control. Furthermore, overexpression of dnIκB-α or p65 knockdown using p65 siRNA attenuated the IL-18-mediated increase in NF-κB (D), but not the constitutively expressed Oct-1 (E), DNA binding activity, and attenuated IL-1β and TNF-α mRNA expression (F). Arrows in panels D and E denote specific DNA-protein complexes. Knockdown of TAK1, IKK-α, and p65 protein levels was confirmed by Western blotting (G). β-Actin was used as an internal control. Furthermore, expression of dominant negative (dn) IRAK, dnTRAF6, dnIKK-γ, dnIκB-α, dnIκB-β, kinase dead IKK-β, and knockdown of MyD88, TAK1, and IKK-α by the respective siRNA, but not empty vectors or scrambled siRNA, significantly attenuated IL-18-mediated IL-1β and TNF-α secretion (H).
IETD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), or a pan-caspase inhibitor (Z-VAD-FMK) significantly attenuated IL-18-induced cell death (Fig. 6D). In addition, knockdown of caspase-8 (knockdown of caspase-8 protein was confirmed by Western blotting, Fig. 6E) and attenuated IL-18-mediated cell death (Fig. 6D).

Activation of caspase-3 leads to the cleavage of various cellular substrates including the proteolytic cleavage of PARP (38). Fig. 6F shows that IL-18, and not neutralized IL-18, induced PARP cleavage, and pretreatment with the pan-caspase inhibitor Z-VAD-FMK attenuated PARP cleavage (Fig. 6G). These results indicate that IL-18 signals via caspase-8-caspase-3-PARP, and induces DNA fragmentation and cell death.

**IL-18 Induces HCMEC Death via BID, Cytochrome c Release, and Caspase-9 Activation**—Cell death by apoptosis occurs via activation of intrinsic and/or extrinsic apoptotic signaling pathways. Activation of caspase-8 cleaves BID, a pro-apoptotic Bcl-2 family member, at aspartate 60 to generate a 15-kDa truncated form that facilitates cytochrome c release from mitochondria (39, 40). Fig. 7A shows that treatment with IL-18 cleaves BID to increase the levels of the 15-kDa truncated form, and this phenomenon is reversed by preincubating the cells with the caspase-8 inhibitor Z-IETD-FMK or the pan-caspase inhibitor Z-VAD-FMK. Furthermore, IL-18 significantly increased cytochrome c release, and both caspase-8 and pan-caspase inhibitors, and caspase-8 knockdown attenuated IL-18-mediated cytochrome c release (Fig. 7B). Upon leakage from mitochondria to the cytoplasm, cytochrome c induces oligomerization of Apaf-1, forms a multimeric complex, and recruits and activates caspase-9 (41). Activation of caspase-9 leads to caspase-3 activation and subsequent cell death (41). Therefore, we investigated the effects of IL-18 on caspase-9 activation. As seen in Fig. 7C, treatment with IL-18 increased cleaved caspase-9 products, and pretreatment with the caspase-9 inhibitor Z-LEHD-FMK, and the pan-caspase inhibitor Z-VAD-FMK pre-
IL-18 activates fas and Fas-L promoter activities via NF-κB activation. Once HCMEC reached 70% confluency, the complete media was replaced with endothelial cell medium + 0.5% BSA. After overnight culture, cells were transiently transfected with 3 μg of fas promoter construct in either orientation (−1739/−19 and −19/−1739) using LipofectAMINE™ reagent. In addition, a deletion construct containing the sequence from −306/−19 that contained a κB-like motif was also analyzed. Transfection with the empty plasmid (pGL-basic) served as a control. Cells were cotransfected with pRl-Benilla to allow correction for variability in transfection efficiency. 24 h after transfection, the media was changed, and the cells were treated with IL-18 (100 ng/ml). *, p < 0.01 versus untransfected; †, p < 0.05 versus IL-18-treated pCMX-transfected cells (A). Furthermore, IL-18 increased κB-driven luciferase activity in cells transfected with pLuc-MCS containing 3 copies of NF-κB site from the fas promoter (B), and overexpression of dnIκBα attenuated IL-18-mediated NF-κB activation. *, p < 0.001 versus pLuc-MCS-transfected controls; †, p < 0.05 versus IL-18-treated pCMX-transfected cells. To demonstrate the effects of IL-18 on Fas-L promoter activity, HCMEC were transiently transfected with 3 μg of Fas-L promoter construct (pLuc-Fas-L). Cells were cotransfected with dnIκBα. 24 h later, cells were treated with IL-18 (100 ng/ml) (C). *, p < 0.01 versus untransfected controls; †, p < 0.05 versus IL-18. In addition, we have also analyzed effects of IL-18 on HCMEC transfected with reporter constructs containing multimers of either the proximal (pLuc-4X-50) or distal (pLuc-3X-530) κB-binding sites from Fas-L (D). *, p < 0.05; **, p < 0.01 versus untransfected controls; †, p < 0.05 versus IL-18 treatment. To further confirm our NF-κB reporter studies (panels B and D), we also performed EMSA using nuclear protein extracts isolated from HCMEC treated with IL-18 (100 ng/ml) for 1 h and double stranded oligonucleotides containing κB-binding sites from fas (lanes 6 and 7), proximal (lanes 8 and 9) and distal (lanes 10 and 11) regions of Fas-L, and consensus NF-κB gel shift oligonucleotides (lanes 4 and 5) from Santa Cruz Biotechnology, Inc. (E). Arrow indicates κB-specific DNA-protein complexes.

Discussion

Results from the present study demonstrate that interleukin-18 activates both intrinsic and extrinsic apoptotic signaling pathways, and induces human cardiac microvascular endothelial cell death. Treatment with IL-18 increased proinflammatory cytokines IL-1β and TNF-α via MyD88, IRAK, TRAF6, IKK, and NF-κB. Furthermore, IL-18 up-regulated proapoptotic Fas, Fas-L, and Bcl-XL expression, increased fas and Fas-L promoter activities in an NF-κB-dependent manner, activated caspases-8, -3, and -9, and inhibited the cellular caspase-8 inhibitor FLIP. In contrast, IL-18 attenuated anti-apoptotic Bcl-2 and Bcl-XL expression. Whereas overexpression of Bcl-2 or inhibition of caspases-8, -3, and -9 attenuated, inhibition of Bcl-2/Bcl-XL expression potentiated IL-18-mediated endothelial cell death.

IL-18 belongs to the IL-1 cytokine family, and has structural similarities with IL-1β (6, 7). Similar to IL-1β, IL-18 is synthesized as a proform and is cleaved by caspase-1 (interleukin-1-converting enzyme (ICE)) to a mature and biologically active form (8). IL-18 acts as a proinflammatory and proapoptotic cytokine, and induces cell death in various immune and non-immune cells (6, 7). We have shown in the present study that treatment with IL-18 induces IL-1β and TNF-α expression via NF-κB activation. IL-18 induced NF-κB activation via MyD88, IRAK, TRAF6, IKK, and IκB degradation. Both IL-1β and TNF-α are negative myocardial inotropes, and induce cell death and amplify the inflammatory cascade (42–47). It is possible that induction of IL-1β and TNF-α during various inflammatory conditions may amplify the inflammatory cas-
IL-18 induces HCMEC death by apoptosis. HCMEC were treated with rhIL-18 (100 ng/ml). At the indicated time periods, adherent and floating cells were collected, pooled, and analyzed for apoptosis using the annexin V-FITC apoptosis detection kit (A). Specificity of IL-18 was confirmed by neutralizing IL-18 with anti-IL-18 antibodies. Normal (preimmune) IgG served as a control (A). *, p < 0.05; **, p < 0.001 versus respective controls; †, p < 0.001 versus IL-18 at 24 h. IL-18-mediated apoptosis was also confirmed by measuring mono- and oligonucleosomal DNA by ELISA using cytoplasmic extracts isolated from HCMEC treated with IL-18 (100 ng/ml; B). HCMEC were preincubated for 1 h with anti-IL-1β, TNF-α, and -IFN-γ neutralizing antibodies (5 μg/ml), treated with TNF-α antisense/scrambled oligonucleotides, or transfected with caspase-1 siRNA expression vector (B). **, p < 0.0001 versus untreated control; †, p < 0.01 versus IL-18. Knockdown of caspase-1 protein was confirmed by Western blotting (C). β-Actin was used as an internal control. Efficacy of TNF-α antisense oligonucleotides was confirmed by pretreating HCMEC with TNF-α antisense oligonucleotides followed by the addition of lipopolysaccharide (1 μg/ml for 24 h). TNF-α levels in culture supernatants were measured by ELISA (D). Transfection with IKK-α or p65 by siRNA, or overexpression of kdIKK-β, dnIKK-γ, dnIkB-α, and dniIkB-β also significantly attenuated IL-18-induced cell death (E). *, p < 0.001 versus untreated control; †, p < 0.05; ††, p < 0.01 versus the respective empty vector-transfected controls. Furthermore, while overexpression of Bcl-2 attenuated, inhibition of Bcl-2/Bcl-X<sub>L</sub> expression potentiated IL-18-induced HCMEC death (F). *, p < 0.0001 versus untreated control; †, p < 0.01 versus IL-18; ‡, p < 0.05 versus IL-18.
cade by inducing IL-18 expression. In fact, we have recently demonstrated that treatment with TNF-α up-regulates IL-18 expression in isolated cardiomyocytes via NF-κB activation (32), indicating a cross-talk between proinflammatory cytokines and IL-18. However, in that study, treatment with TNF-α also induced IL-18-binding protein expression in a delayed and persistent fashion. IL-18-binding protein acts as a soluble decoy receptor, and neutralizes IL-18, and thereby limits the proinflammatory effects of IL-18 (48). Whether IL-18 induces IL-18-binding protein expression in cardiac endothelial cells is yet to be investigated.

Both IL-1β and TNF-α have previously been shown to induce endothelial cell apoptosis through induction of iNOS, iNOS-mediated nitric oxide generation, and activation of death receptor-mediated pro-apoptotic signaling pathways (46, 49). Recently, IL-18 has been shown to synergize with TNF-α in inducing hepatic endothelial cell death (50). Although IL-18 induces IL-1β and TNF-α expression (Fig. 2), neutralization of IL-1β or TNF-α or inhibition of TNF-α by antisense oligonucleotides failed to inhibit IL-18-mediated cell death. In addition, neutralization of IL-1β and TNF-α together also failed to modulate IL-18-induced endothelial cell death (data not shown). Similarly, neutralization of IFN-γ, which is critical in mediating some of the biological effects of IL-18, failed to in-

**Fig. 6.** IL-18 activates caspase-8, caspase-3, and PARP and inhibits FLIP. HCMEC were treated with IL-18 (100 ng/ml) for up to 16 h. Activation of caspase-8, caspase-3, and PARP was analyzed by Western blotting. Similarly, FLIP (FLICE inhibitory protein) levels were also analyzed by Western blotting. Our results indicate that treatment with IL-18 activates caspase-8 as indicated by reduced levels of full-length caspase-8 and increased levels of the active cleaved products (p43/p41, p18) (A). On the other hand, treatment with IL-18 significantly inhibited the cellular caspase-8 inhibitor, FLIP (B, densitometric values from three different experiments are shown in the lower panel; *p < 0.01 versus untreated control). IL-18 also induced caspase-3 activation as seen by increased levels of the active cleaved p17/p19 form (C). Furthermore, pretreatment for 2 h with caspase-8 (Z-IETD-FMK), caspase-3 (Z-DEVD-FMK), or pan-caspase inhibitor (Z-VAD-FMK) as well as caspase-8 knockdown using caspase-8 siRNA expression vector, attenuated IL-18-induced HCMEC death (D). Z-FA-FMK used as a negative control, or Me2SO used as a solvent control, failed to modulate IL-18-induced cell death. *, p < 0.001 versus untreated; †, p < 0.05; ††, p < 0.01 versus respective controls. Knockdown of caspase-8 was confirmed by Western blotting (E). Treatment with IL-18-activated PARP, as seen by increased levels of cleaved PARP in the nuclear protein extracts (F), and pretreatment with caspase-3 and pan-caspase inhibitors inhibited IL-18-mediated PARP activation (G).
**FIG. 7.** IL-18 induces cell death via activation of the intrinsic (mitochondrial) apoptotic pathway. HCMEC were treated with IL-18 (100 ng/ml) for 16 h. Activation of BID and caspase-9 was analyzed by Western blotting. Cytochrome c levels in the cytoplasmic and mitochondrial fractions were analyzed by ELISA as described under “Experimental Procedures.” Our results indicate that treatment with IL-18 activates BID in HCMEC (A), and specific inhibition of caspase-8 or pan-caspase inhibition attenuated IL-18-mediated BID activation (A). Treatment with IL-18 increased cytochrome c release into the cytoplasm (B), and both caspase-8 (Z-IETD-FMK) and pan-caspase inhibitors (Z-VAD-FMK) and caspase-8 knockdown inhibited IL-18-mediated cytochrome c release (B). Cyto, cytoplasmic fraction; Mito, mitochondrial fraction. *, p < 0.001 versus untreated; †, p < 0.0001 versus IL-18 alone. Furthermore, treatment with IL-18 activated caspase-9 as seen by increased cleaved products (p37 and p35), and pretreatment with caspase-9 inhibitor (Z-LEHD-FMK) attenuated IL-18-induced caspase-9 activation (C). Schematic representation of possible signal transduction pathways involved in IL-18-mediated NF-κB activation, proinflammatory and proapoptotic gene expression, and HCMEC death (D).
hibit or potentiate IL-18-mediated cell death. Furthermore, knockdown of caspase-1, which prevents proteolytic cleavage of pro-IL-18 and pro-IL-1β to their mature biologically active forms, failed to modulate IL-18-induced cell death, indicating that the observed proapoptotic effects are because of the exogenously added IL-18, and are not mediated by intermediaries such as IL-1β, TNF-α, or IFN-γ.

In addition to its stimulatory effects on proinflammatory cytokine expression, treatment with IL-18 increased the proapoptotic Fas and Fas-L expression. Using promoter-reporter constructs, we clearly demonstrate that IL-18 activates both Fas and Fas-L promoter activities via NF-κB activation. Fas, the most extensively studied and a well characterized death receptor, belongs to the TNF receptor superfamily. Binding of Fas-L to Fas triggers aggregation of Fas receptors with the recruitment of FADD (Fas-associated death domain) to their cytoplasmic portions (51, 52). FADD then activates procaspase-8 by interacting with its death domain. The resulting cleavage and activation of caspase-8 triggers various downstream proapoptotic signaling pathways (51–53). In the present study we demonstrate that treatment with IL-18 activates caspase-8, and pretreatment with Z-IETD-FMK or the pan-caspase inhibitor Z-VAD-FMK inhibited IL-18-mediated caspase-8 activation. FLIP, a cytoplasmic protein with homology to caspase-8 lacks a cysteine residue that is essential for proteolysis, and acts in a dominant negative manner to inhibit caspase-8 activation (37, 54, 55). Endothelial cells express high levels of FLIP, and are therefore less susceptible to apoptosis (56). Our results in Fig. 6B indicate that endothelial cells express high levels of FLIP at basal conditions, and treatment with IL-18 significantly reduced its expression levels. This decrease in FLIP expression also might have been a contributing factor to IL-18-mediated endothelial cell apoptosis. Upon activation, caspase-8 cleaves pro-caspase-3, a downstream effector caspase, to an active caspase-3. In fact, we demonstrate that treatment with IL-18 activates caspase-3, and caspase-3-dependent PARP cleavage and DNA fragmentation (Fig. 6). Of note, caspase-3 has been shown to degrade mature IL-18 to inactive metabolites (57). It is possible that once the apoptotic machinery is activated, persistent expression of IL-18 may not be necessary to induce cell death.

In addition to activating the extrinsic pathway, IL-18 also regulates the intrinsic pathway as seen by increased release of cytochrome c into the cytoplasmatic extracts. Caspase-8, which is at the interphase between the extrinsic and the intrinsic pathways, activates BID forming truncated BID (tBID; carboxy-terminal region of BID). Cleaved BID then translocates to the mitochondria and induces cytochrome c release. Cytochrome c then forms an apoptosome-dATP-dependent complex by interacting with Apaf-1 (58–61). This complex leads to the activation of caspase-9 (61). We show that treatment with IL-18 activates BID forming a 15-kDa cleaved active form (Fig. 7). Likewise, treatment with IL-18 induces caspase-9 activation, which then cleaves and activates caspase-3. Pretreatment with Z-IETD-FMK, a specific caspase-8 inhibitor or Z-VAD-FMK, a pan-caspase inhibitor, prevented IL-18-mediated BID cleavage and cytochrome c release, confirming that IL-18 also signals via the intrinsic (mitochondrial) pathway.

Our studies also indicate that treatment with IL-18 induces Bcl-XL expression, indicating a shift in the regulatory balance of the Bcl-2 family proteins favoring apoptosis, and further supports activation of mitochondrial-dependent pro-apoptotic pathways. Alternative splicing results in the formation of either the proapoptotic Bcl-XS or the anti-apoptotic Bcl-XL (62). Whereas Bcl-XL has both size and structural similarities to Bcl-2, Bcl-XL lacks 63 highly conserved amino acids that span both the BH1 and BH2 domains present in the anti-apoptotic members of the Bcl-2 family (62). Furthermore, stable expression of Bcl-XL antagonizes the anti-apoptotic properties of Bcl-2 and Bcl-XL, inducing cell death (63). Our data indicate that following IL-18 treatment Bcl-XL expression is markedly increased. In contrast, Bcl-2 expression is inhibited. However, Bcl-XL expression is not modulated by IL-18. Whereas overexpression of Bcl-2 attenuated, inhibition of Bcl-2/Bcl-XL by bispecific antiensense oligonucleotides potentiated IL-18-mediated cell death. These results indicate that treatment with IL-18 shifts the balance in favor of apoptosis by increasing Bcl-XL/Bcl-XL/Bcl-XL ratios.

Our studies have several important implications. (i) IL-18 expressed locally by endothelial cells may initiate tissue injury by inducing cell death. (ii) Endothelial cell-mediated IL-18 expression may induce chemokine expression, attract and activate specific subsets of immune cells to the site of injury or inflammation. For example, IL-18 has been shown to induce IL-8 and MIP-2 expression, and attract and activate polymorphonuclear leukocytes (9, 10, 64). Polymorphonuclear leukocytes play an important role in post-ischemic myocardial pathobiology (65–67). (iii) Endothelial cell-mediated IL-18 expression may induce adhesion molecule expression, promoting adhesion of immune and inflammatory cells. For example, IL-18 has been shown to induce intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 expression (19). (iv) Endothelial cell-mediated IL-18, via autocrine and paracrine mechanisms, may induce IL-1β, TNF-α, and iNOS expression in myocardial constituent cells (6, 7, 68). These cytokines have proinflammatory, proapoptotic, and negative myocardial inotropic effects. The cross-talk between IL-18 and these proinflammatory molecules may further amplify the inflammatory cascade. Therefore, targeting IL-18 expression or NF-κB activation may attenuate chemokine, cytokine, and adhesion molecule expression, inhibit inflammatory cell infiltration, attenuate cell death, and reduce tissue injury.

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Activation of Intrinsic and Extrinsic Proapoptotic Signaling Pathways in Interleukin-18-mediated Human Cardiac Endothelial Cell Death
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J. Biol. Chem. 2004, 279:20221-20233.
doi: 10.1074/jbc.M313980200 originally published online February 11, 2004

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