Glucocorticoids Induce Osteocyte Apoptosis by Blocking Focal Adhesion Kinase-mediated Survival

**EVIDENCE FOR INSIDE-OUT SIGNALING LEADING TO ANOIKIS**

Received for publication, December 13, 2006, and in revised form, May 14, 2007

Published, JBC Papers in Press, June 20, 2007, DOI 10.1074/jbc.M611435200

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Bone fragility induced by chronic glucocorticoid excess is due, at least in part, to induction of osteocyte apoptosis through direct actions on these cells. However, the molecular mechanism by which glucocorticoids shorten osteocyte life span has remained heretofore unknown. We report that apoptosis of osteocytic MLO-Y4 cells induced by the synthetic glucocorticoid dexamethasone is abolished by the glucocorticoid receptor antagonist RU486, but not by inhibition of protein or RNA synthesis. Dexamethasone-induced apoptosis is preceded by a decrease in the number of cytoplasmic processes, an indicator of cell detachment. In addition, the focal adhesion kinase FAK prevents dexamethasone-induced apoptosis, whereas the FAK-related kinase Pyk2 increases the basal levels of apoptosis. Dexamethasone-induced apoptosis is also prevented in cells expressing kinase-deficient or phosphorylation-defective (Y402F) dominant negative mutants of Pyk2. Consistent with the requirement of tyrosine 402, dexamethasone induces rapid Pyk2 phosphorylation in this residue. Moreover, knocking down Pyk2 expression abolishes apoptosis and cell detachment induced by dexamethasone, and transfection with human Pyk2 rescues both responses. Furthermore, induction of apoptosis as well as cell detachment by dexamethasone is abolished by inhibiting the activity of JNK, a recognized downstream target of Pyk2 activation. These results demonstrate that glucocorticoids promote osteocyte apoptosis via a receptor-mediated mechanism that does not require gene transcription and that is mediated by rapid activation of Pyk2 and JNK, followed by inside-out signaling that leads to cell detachment-induced apoptosis or anoikis.

Glucocorticoids, produced and released by the adrenal glands in response to stress, regulate numerous physiological processes in a wide range of tissues (1, 2). Among many other effects, these hormones exert profound immunosuppressive and anti-inflammatory actions and induce apoptosis of many cell types, including T lymphocytes and monocytes. Because of these properties, glucocorticoids are extensively used for the treatment of immune and inflammatory conditions, the management of organ transplantation, and as components of chemotherapy regimens for hematological cancers. However, long-term use of glucocorticoids is associated with severe adverse side effects manifested in several organs. In particular, prolonged use of these drugs leads to a dramatic loss of bone mineral and strength, similar to endogenous elevation of glucocorticoids (3–6). Evidence accumulated during the past few years has indicated that increased prevalence of apoptosis of osteocytes (and osteoblasts) is associated with the glucocorticoid-induced bone fragility syndrome (7–9). This pro-apoptotic effect results from direct actions of the steroids on cells of the osteoblastic lineage. Indeed, the pro-apoptotic effect of glucocorticoids is readily demonstrable in cultured osteocytes and osteoblasts (10, 11). Furthermore, transgenic mice overexpressing 11β-hydroxysteroid dehydrogenase type 2, an enzyme that inactivates glucocorticoids, in osteocytes and osteoblasts are protected from glucocorticoid-induced bone fragility (8).

The mechanism of glucocorticoid action involves binding to the glucocorticoid receptor (GR),2 conformational changes, and nuclear translocation of the ligand-bound receptor, followed by cis or trans interactions with DNA and thereby induction or repression of gene transcription (1, 2). In addition, glucocorticoids exert actions independently of changes in gene transcription. Such actions include modulation of the activity of intracellular kinases like the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and proline-rich tyrosine kinase 2 (Pyk2) (12–17). Pyk2, also known as related adhesion focal tyrosine kinase, cellular adhesion kinase β, or calcium-dependent tyrosine kinase (18, 19), is a member of the focal adhesion kinase (FAK) family of nonreceptor tyrosine kinases. Although Pyk2 and FAK are highly homologous, these proteins exhibit opposite effects on cell fate. Thus, whereas FAK activation leads to cell spreading and survival (20), Pyk2 induces reorganization of the cytoskeleton, cell detachment, and apoptosis (18, 20). Consistent with these lines

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2 The abbreviations used are: GR, glucocorticoid receptor; ROS, reactive oxygen species; ERKs, extracellular signal regulated kinases; Pyk2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; GFP, green fluorescent protein; nGFP, GFP targeted to the nucleus; nRFP, RFP targeted to the nucleus; WT, wild type; K−, kinase deficient; PI3K, phosphatidylinositol 3-kinase; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.

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of evidence, we have recently demonstrated that mechanical stimulation promotes osteocyte survival by activating FAK (21). We now report that glucocorticoids promote osteocyte apoptosis by activating Pyk2 and JNK, hence opposing FAK-induced survival. These changes lead to cell detachment-induced apoptosis (anoikis). This action of glucocorticoids is exerted via a receptor-mediated mechanism, but it is independent of new gene transcription.

EXPERIMENTAL PROCEDURES

Materials—The synthetic glucocorticoid dexamethasone, etoposide, cycloheximide, actinomycin D, RU486, SB203580, wortmannin, EGTA, and gadolinium chloride were purchased from Sigma; SP600125 and nifedipine from EMD Biosciences, Inc. (San Diego, CA); Asp-Glu-Val-Asp-aldehyde (DEVD) and thapsigargin from Biomol Research Labs, Inc. (Plymouth Meeting, PA); PD98059 from New England Biolabs (Beverly, MA); PP1 from BioSource International (Camarillo, CA); [3H]leucine or [3H]uridine from Amersham Biosciences; and BAPTA-AM and phalloidin-Alexa Fluor 555 from Molecular Probes (Carlsbad, CA).

DNA Constructs and Transient Transfections—pCDA3, yellow fluorescent protein (YFP)-DEV caspase 3 sensor and green fluorescent protein (GFP) were purchased from Clontech. The plasmids encoding nuclear targeted GFP (nGFP) and red fluorescent protein (nRFP) were previously described (11, 22). Human wild type (WT) FAK was provided by S. Aruffo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) (23). Murine WT, kinase-deficient (K−), and autophosphorylation deficient (Y402F) Pyk2 mutants were provided by W.-C. Xiong (University of Alabama, Birmingham, AL) (18). Human WT and K− Pyk2 mutant fused to GFP (Pyk2-GFP) were provided by D. Sancho (Universidad Autónoma de Madrid, Madrid, Spain) (24). Dominant negative and constitutively active MEK were provided by N. Ahn (University of Colorado, Boulder, CO (25)). Wild type and dominant negative JNK were provided by E. R. Levin (University of California at Irvine, Long Beach, CA) (26, 27) and ΔMEK K1, by N. R. Bhat (Medical University of South Carolina, Charleston, SC) (28). All the constructs used in this study have been shown to produce functional proteins. Cells were transiently transfected with a total amount of DNA of 0.1 μg/cm2 using Lipofectamine Plus (Invitrogen) as previously described (22, 29). The efficiency of transfection was ~60%.

Cell Culture—Wild type MLO-Y4 osteocytic cells derived from murine long bones and MLO-Y4 cells stably transfected with nGFP were cultured as previously described (11, 30).

Inhibition of RNA or Protein Synthesis—MLO-Y4 cells were incubated with 1 μCi/ml [3H]leucine or [3H]uridine for 2.5 h, followed by addition of 1 μM cycloheximide or 2 μM actinomycin D. After 7.5 h, cells were scraped off the plates, protein and RNA were precipitated with ice-cold 5% trichloroacetic acid, and radioactivity was quantified. Protein concentration was determined using a detergent compatible Bio-Rad kit. [3H]Leucine or [3H]uridine incorporation was expressed as counts/min/μg of protein.

Quantification of Apoptotic Cells—Apoptosis was induced in semi-confluent cultures (less than 75% confluence) by treat-
to polyvinylidene difluoride membranes. Immunoblottings were performed using a rabbit anti-phosphorylated Pyk2 antibody (BioSource, Camarillo, CA) or rabbit anti-Pyk2 antibody that recognizes both the human and murine protein (Upstate, Charlottesville, VA). Human GFP-Pyk2 expression was detected using a mouse monoclonal anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA). β-Actin was detected using a mouse monoclonal antibody (Sigma). After incubation with primary antibodies, blots were exposed to anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) and developed using a chemiluminescence substrate (Pierce). The intensity of the bands was quantified using the Versadoc Imaging system (Bio-Rad).

Real Time PCR—Total RNA was obtained using Ultraspec RNA isolation reagent (Biotex Laboratories, Houston, TX). Reverse transcription was performed using the High Capacity cDNA Archive Kit. Primers and probes for the housekeeping gene 

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(probe, 5'-TCCAGACCCGATACT-3', forward primer, CCCAGGATGCGAGCAT and reverse primer, CCGAATGTGCATATGCCGTAT) (Assay-by-Design service) and TaqMan Gene Expression Assay for murine Pyk2 (Mm00552840_m1) were used. The PCR was performed using 20 μl of Gene Expression Assay Mix TaqMan Universal Master Mix containing 80 ng of each cDNA template in triplicates, using an ABI 7300 Real Time PCR system. The -fold change in expression was calculated using the ΔΔCt comparative threshold cycle method. All the reagents were from Applied Biosystems.

Image Acquisition—Fluorescent images were collected on an Axioplan 2 inverted microscope or an Axioplan 2 microscope (Carl Zeiss Light Microscopy, Gottingen, Germany) with a LD A-Plan, ×32/0.40 lens and a low light camera (Polaroid DMC Le, Polaroid Corp., Cambridge, MA), using filter sets for GFP, YFP, or RFP, and the Image-Pro Plus acquisition software (Media Cybernetics, Silver Spring, MD). Confocal images were obtained with an Axiocorvet LSM410 inverted confocal microscope (Zeiss LSM410) using the 568-nm line of an Argon-Krypton laser and a 590-nm long pass emission filter.

Statistical Analysis—Data were analyzed by one-way analysis of variance, and the Student-Newman-Keuls method was used to estimate the level of significance of differences between means.

RESULTS

Glucocorticoid-induced Apoptosis Does Not Require New Gene Transcription and It Is Mediated by the GR—We and others have previously demonstrated that glucocorticoids induce apoptosis of osteocytic MLO-Y4 cells (11, 29, 35). We have used this cell model herein to gain insight into the mechanism of the pro-apoptotic effect of glucocorticoids. We found that dexamethasone induced apoptosis even in the presence of the protein synthesis inhibitor cycloheximide or the RNA synthesis inhibitor actinomycin D (Fig. 1A); even though [3H]leucine or [3H]uridine incorporation into proteins or RNA were effectively blocked (21.4 ± 0.8 and 9.6 ± 5.1% of control cultures, respectively). The pro-apoptotic effect of etoposide on osteocytic cells, used here for comparison, was similarly unaffected by the presence of the protein or RNA synthesis inhibitors (Fig. 1A).

To determine whether the GR was required for glucocorticoid-induced apoptosis in osteocytic cells, we examined the effect of dexamethasone in the presence of RU486, a GR antagonist (36). Apoptosis induced by 6 h treatment with dexamethasone was absent in cells pre-treated with RU486, whereas apoptosis induced by etoposide was not affected, demonstrating the specificity of the receptor antagonist on the glucocorticoid effect (Fig. 1B).

Microscopic examination revealed a profound effect of dexamethasone on the shape of MLO-Y4 cells. Based on this observation, we set up experiments to quantify the phenomenon by evaluating the number of cytoplasmic processes, as an indicator of cell rounding and detachment. Six-h treatments with dexamethasone increased the percentage of cells with ≤3 cytoplasmic prolongations (C). Bars represent mean ± S.D. of triplicate determinations. *, p < 0.05 versus vehicle-treated cultures.

Cell Detachment Precedes Induction of Osteocyte Apoptosis by Glucocorticoids—To determine whether cell detachment preceded or followed dexamethasone-induced apoptosis, time course experiments were performed in which apoptosis and cell detachment were evaluated in the same cultures. Apoptosis induced by dexamethasone was first detected 3 h after the addition of the hormone (Fig. 2, A and B), whereas the increase in the percentage of cells exhibiting ≤3 cytoplasmic processes was
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Retraction of osteocyte cytoplasmic processes and cell rounding induced by dexamethasone was also revealed by actin reorganization visualized by confocal microscopy of cells stained with Alexa Fluor 555-conjugated phalloidin (Fig. 3B). Indeed, the glucocorticoid-induced disruption of stress fibers and formation of peripheral actin rings as early as 1 h and these changes were maintained throughout the 24-h culture. In contrast, cells treated with etoposide only showed changes in actin filament organization after 24 h. These findings indicate that changes in cell shape precede glucocorticoid-induced cell detachment. In contrast, cell detachment induced by etoposide occurred subsequent to apoptosis induced by this agent.

The Mechanism of Glucocorticoid-induced Apoptosis Involves Focal Adhesion Kinases—Cell attachment and survival depend on the formation of focal adhesions and activation of focal adhesion-associated proteins, such as FAK (37, 38). We have previously shown that phosphorylation of FAK is required for the anti-apoptotic effect of mechanical stimulation on osteocytic cells; and that osteocytic cells overexpressing FAK are resistant to the pro-apoptotic effect of dexamethasone (21). Based on this and on evidence indicating that FAK and Pyk2 have opposite effects on cell survival (17, 18, 20), we investigated whether Pyk2 was involved in the effect of glucocorticoids on osteocytic cells. Dexamethasone induced rapid phosphorylation of Pyk2 in tyrosine 402, which was maximal at 10 min and declined to basal levels by 30 min (Fig. 4A). Moreover, consistent with our previous observations (21), dexamethasone induced apoptosis in cells expressing empty vector but not in cells overexpressing FAK (Fig. 4B). In contrast, whereas dexamethasone was able to induce apoptosis in cells overexpressing wild type Pyk2, the pro-apoptotic effect of the glucocorticoid was abolished in cells expressing either a Pyk2 mutant that lacks kinase activity (K−) of murine or human origin (Fig. 4, B and C) or a murine Pyk2 mutant that cannot be phosphorylated in tyrosine 402 (Y402F) (Fig. 4B).

Remarkably, cells expressing wild type or K− Pyk2 exhibited a higher level of basal apoptosis than vector-transfected cells. This effect was observed even when 10 times lower plasmid concentrations were transfected into the cells, leading to lower expression of the proteins, suggesting that it was not due to supra-physiological levels of Pyk2 expression (Fig. 4, B−D). Increased basal apoptosis was not observed in cells transfected with Y402F Pyk2. In contrast to their effect on dexamethasone-induced apoptosis, overexpression of FAK, or wild type or mutated Pyk2 did not affect the pro-apoptotic effect of etoposide (Fig. 4, B and C).

We next investigated the requirement of Pyk2 expression for dexamethasone-induced apoptosis and changes in cell shape, by silencing the expression of murine Pyk2 with small interference RNAs. Pyk2 mRNA and protein expression was greatly
reduced compared with mock-transfected cells or cells transfected with the negative control small interfering RNA for lamin, as determined by real time reverse transcriptase-PCR and Western blotting, respectively (Fig. 5, A and B). Transfection with human wild type or K+/H11002 Pyk2 fused to GFP recovered Pyk2 expression, as evidenced by Western blot analysis using anti-Pyk2 and anti-GFP antibodies (Fig. 5B) and by fluorescence microscopy (Fig. 5C, upper panels). Cell detachment was quantified by enumerating the number of cytoplasmic processes using a filter set for GFP. To allow simultaneous assessment of apoptosis, cells were co-transfected with rRFP and apoptotic cells were quantified using a filter set for RFP (Fig. 5C, lower panels).

Dexamethasone did not induce apoptosis or cell detachment in cells in which Pyk2 expression had been knocked down (Fig. 5, D and E). Moreover, transfection of human wild type Pyk2, but not K+/H11002 Pyk2, rescued both apoptosis and cell detachment induced by the glucocorticoid. On the other hand, silencing the control protein lamin did not affect apoptosis or cell detachment induced by dexamethasone. In addition, transfection of wild type or K+/H11002 Pyk2 not only increased basal levels of apoptosis confirming the results of Fig. 4C, but also increased the percentage of cells with ≤3 cytoplasmic processes (Fig. 5E). Knocking down or overexpressing Pyk2 did not modify the response of osteocytic cells to etoposide.

Taken together, these findings indicate that Pyk2 expression, its kinase activity, and tyrosine 402 are required for glucocorticoid-induced apoptosis and cell detachment. Furthermore, these results demonstrate that overexpression of Pyk2 increases osteocytic cell detachment and apoptosis; and that this effect is independent of Pyk2 kinase activity, although it requires tyrosine 402 phosphorylation.

Glucocorticoid-induced Apoptosis Requires Ca2+/H11001 Entry through Stress-activated Ca2+/H11001 Channels

In line with the requirement of Pyk2 for the pro-apoptotic effect of glucocorticoids and previous evidence indicating that elevation of cytosolic Ca2+/H11001 leads to Pyk2 activation (20), dexamethasone did not induce osteocyte apoptosis in cells in which intracellular Ca2+/H11001 was chelated with BAPTA-AM (Fig. 6A). Depletion of intracellular stores with thapsigargin, however, did not affect the pro-apoptotic action of dexamethasone. In contrast, chelation of extracellular calcium with EGTA abolished the pro-apoptotic effect of dexamethasone. In addition, blockade of L-type voltage-sensitive calcium channels with nifedipine had no

FIGURE 3. Glucocorticoid-induced osteocyte apoptosis is preceded by cell detachment. A, cells were incubated with vehicle (veh), dexamethasone (dex), or etoposide (etop) for the indicated times. Cell detachment was quantified in MLO-GFP cells as detailed under “Experimental Procedures.” Representative images of MLO-GFP or MLO-Y4 cells stained with H&E show the morphologic changes observed after 6 h of treatment. B, actin filament reorganization was visualized in MLO-Y4 cells by confocal microscopy of cells stained with phalloidin-Alexa Fluor 555. C, MLO-GFP cells were treated with vehicle or DEVD before addition of the pro-apoptotic agents. Cell detachment was quantified as in A. Bars represent mean ± S.D. of triplicate determinations.
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A

|     | pTyr402 Pyk2 | Pyk2 |
|-----|--------------|------|
|     | pPyk2/Pyk2   |
| 0   | 0            | 1.0  |
| 5    | 0.9          | 1.4  |
| 10   | 0.8          | 2.3  |
| 15   | 0.6          | 1.5  |
| 30   | 0.0          | 1.2  |

B

|     | % apoptotic cells |
|-----|------------------|
| vector | FAK | wt | K | Y402F |
| 5x10^{-3} µg/cm² | 0% | 5% | 15% | 20% |
| 5x10^{-3} µg/cm² | 0% | 5% | 15% | 20% |

C

|     | % apoptotic cells |
|-----|------------------|
| vector | wt | K | Y402F |
| 5x10^{-3} µg/cm² | 0% | 5% | 15% |
| 5x10^{-5} µg/cm² | 0% | 5% | 15% |

D

|     | mPyk2 | hPyk2-GFP |
|-----|-------|-----------|
| vector | FAK | wt | K | Y402F |
| 5x10^{-3} µg/cm² | 0% | 5% | 15% | 20% |
| 5x10^{-5} µg/cm² | 0% | 5% | 15% | 20% |

FIGURE 4. FAK prevents, whereas Pyk2 kinase activity and phosphorylation are required for, glucocorticoid-induced apoptosis. A, Pyk2 phosphorylation in tyrosine 402 was determined by Western blot analysis of MLO-Y4 cells treated with dexamethasone for the indicated times. A representative experiment and the fold changes (± S.D.) in five independent experiments are shown. B–D, cells were transiently transfected with the indicated amounts of Pyk2 along with nGFP (for murine Pyk2) or nRFP (for human Pyk2-GFP). Forty hours after transfection, apoptosis was assayed as detailed under “Experimental Procedures.” *, p < 0.05 versus vehicle-treated cultures for each construct; #, p < 0.05 versus vector-transfected, vehicle-treated cultures. D, Pyk2 levels were determined by Western blotting in MLO-Y4 cells transiently transfected with wild type and mutated murine Pyk2 or human Pyk2-GFP. Western blotting for β-actin shows sample loading. Bars represent mean ± S.D. of triplicate determinations.

effect, whereas inhibition of stress-activated channels with gadolinium abolished glucocorticoid-induced apoptosis. Moreover, BAPTA, EGTA, and gadolinium prevented Pyk2 phosphorylation induced by dexamethasone (Fig. 6B).

Pyk2 activation has been shown to result in activation of several kinases, including Src, ERKs, and PI3K, as well as the pro-apoptotic members of mitogen-activated protein kinase family p38 and JNK (20, 39, 40). We therefore investigated which of these kinases was involved in the pro-apoptotic effect of glucocorticoids on osteocytes. To this end, we used pharmacologic inhibitors at concentrations known to effectively inhibit the corresponding kinases in osteocytic and osteoblastic cells (11, 21, 29, 41). Inhibition of JNK with SP600125 blocked dexamethasone-induced osteocyte apoptosis (Fig. 6C). On the other hand, inhibitors of Src, ERKs, PI3K, or p38 kinases had no effect downstream of Pyk2 activation by dexamethasone.

Taken together, these findings strongly suggest that glucocorticoids activate Pyk2 by inducing Ca^{2+} entrance from the extracellular space leading to a rapid rise in intracellular Ca^{2+}. Pyk2 activation, in turn, leads to activation of JNK, followed by cell detachment and apoptosis.

DISCUSSION

Both cell survival and attachment are controlled by the focal adhesions, sites at the plasma membrane in which integrins interact with the extracellular matrix and with intracellular structural and catalytic molecules (42–44). Signaling mediated by integrins is bidirectional. Thus, extracellular matrix proteins induce integrin engagement and activate intracellular signaling (referred to as outside-in signaling). In turn, activation of intra-
cellular signaling or changes in the composition of the focal adhesions regulates the interaction of integrins with extracellular matrix proteins (referred to as inside-out signaling) (45, 46). Whereas association of integrins with the extracellular matrix leads to survival, loss of this interaction causes detachment-induced apoptosis or anoikis (44). The findings reported herein together with previous observations of ours demonstrate that integrin engagement maintains osteocyte survival and that glucocorticoids oppose this integrin/FAK-dependent anti-apoptotic signaling by activating Pyk2 and JNK. This rapid intracellular signaling is followed by inside-out signaling that causes osteocyte detachment and leads to anoikis.

Although not directly investigated in the present study, it is likely that glucocorticoids promote apoptosis of osteoblasts by a similar mechanism than the one described here for osteocytes. Indeed, survival of both osteoblasts and osteocytes is maintained by their interaction with extracellular matrix proteins, as indicated by the fact that neutralizing antibodies to the extracellular matrix protein fibronectin or inhibitors of metalloproteinases induce osteoblast apoptosis (47–49). Moreover, transgenic mice expressing collagenase-resistant collagen type-I exhibit increased prevalence of osteoblast and osteocyte apoptosis (50), and, osteocyte apoptosis is also elevated in metalloproteinase-2 null mice (51). Therefore, cellular interactions with intact or cryptic sites of extracellular matrix proteins are required to maintain osteocyte and osteoblast viability.

Osteocytes are tethered through integrins to the lacunar and canalicular walls by fibers composed of proteoglycans (52). It has been proposed that fluid movement in the canaluli resulting from mechanical loading generates a drag force that shortens the distance between osteocyte membranes and the canalicular wall, inducing strain at the membrane level (53). We have recently demonstrated that bone loading is required to maintain osteocyte viability (54), and mechanical forces are transduced into intracellular survival signaling by integrins and a signalsome comprising cytoskeletal and catalytic proteins including FAK (21). The current studies support the notion that osteocyte fate is controlled by the balance between FAK and matrix.
Pyk2. Thus, FAK overexpression abolishes glucocorticoid-induced apoptosis of osteocytes. Conversely, Pyk2 overexpression leads to osteocyte apoptosis even in the absence of glucocorticoids. Consistent with these findings, FAK activation prevents anoikis in different cell types (55–57); and Pyk2 overexpression induces apoptosis of fibroblasts (18). Similar to our results, Xiong and Parsons (18) found that the kinase-deficient Pyk2 (K−) is also able to stimulate apoptosis. The mechanism of this phenomenon is unknown. One possibility is that by overexpressing either form of Pyk2, the ratio Pyk2/FAK at the focal adhesions increases, thereby counteracting the pro-survival effect of FAK. The inability of the unphosphorylatable Y402F Pyk2 to induce apoptosis might result from deficient targeting of the mutant to the focal adhesions or its failure to displace FAK.

Our findings indicate that one of the consequences of Pyk2 activation is the activation of the pro-apoptotic kinase JNK. However, it is likely that changes in the ratio between Pyk2 and FAK activity induced by glucocorticoids favor apoptosis by additional mechanisms, such as reducing the activity of ERKs or PI3K, downstream targets of FAK with recognized pro-survival effects on osteoblastic cells (11, 21, 22). These changes in signaling downstream of focal adhesion kinases induced by glucocorticoids, combined with down-regulation of genes that prolong survival, such as interleukin-6, insulin growth factors, transforming growth factor β, collagenase type I, and integrin β1 (1, 58–61), could result in the increased prevalence of osteocyte and osteoblast apoptosis observed in vivo.

Consistent with our findings in osteocytes, glucocorticoids modulate intracellular signaling in other cells through rapid actions independent of transcriptional effects, but dependent on the GR. Specifically, ERKs, PI3K, p38, and JNK kinases are activated or inhibited by these agents depending on the cell type (2, 13, 62–66). In addition, glucocorticoids activate Pyk2. Moreover, similar to our findings in osteocytic cells, overexpression of a kinase-inactive Pyk2 mutant abolishes glucocorticoid-induced apoptosis of myeloma cells (17).

Support for the in vivo relevance of the mechanism described herein has been provided by studies in transgenic mice expressing a dimerization-deficient GR that lacks the ability to bind to DNA (67). In line with the evidence of the present report that glucocorticoid-induced apoptosis of osteocytes (and most likely osteoblasts) is mediated through a transcription-independent mechanism, Tuckermann et al. (67) have found that DNA binding of the GR is indeed dispensable for the deleterious effect of glucocorticoids on the skeleton of these mice.

Earlier work of ours (22, 41) has demonstrated that non-genotropic actions of the receptors for estrogen or vitamin D can be dissociated from transcriptional effects using synthetic ligands. This knowledge, along with the findings reported herein, raises the hope that one may be able to design in the future anti-inflammatory glucocorticoid analogs incapable of activating Pyk2; and hence spare the skeleton from the adverse effect of classical glucocorticoids.

The precise mechanism by which glucocorticoids activate Pyk2 in osteocytes is unclear. However, our findings implicate a raise in intracellular Ca2+ via stress-activated channels. In line with these findings, Pyk2 activation by stress inducers is Ca2+ dependent (16). Moreover, Pyk2 is activated by agents that increase cytoplasmic Ca2+, such as thapsigargin, Ca2+ iono-
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In summary, we describe herein a novel pathway triggered by glucocorticoids in osteocytes mediated by the GR. It involves activation of rapid intracellular signaling leading to detachment-induced apoptosis. Interference with this pathway may prove beneficial for counteracting the adverse skeletal impact of glucocorticoid- or aging-induced osteocyte apoptosis leading to bone fragility.

Acknowledgments—We thank Kanan Vyas and William Webb for technical assistance and members of the University of Arkansas Medical Sciences Center for Osteoporosis and Metabolic Bone Diseases for insightful suggestions.

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In contrast to their pro-apoptotic effect on osteocytes and osteoblasts, glucocorticoids prolong the lifespan of osteoclasts (77, 78), but the mechanism of this effect remains unknown. Pyk2 is critical for osteoclast function as its phosphorylation in tyrosine 402 is required for formation of the sealing zone and initiation of bone resorption by the osteoclast (79, 80). In view of this evidence, the findings of the present report raise the possibility that the pro-survival effects of glucocorticoids on osteoclasts result from activation of Pyk2 and increased adhesion, thereby prolonging the resorbing activity of osteoclasts. Future studies are required to examine this possibility.

Finally, recent evidence indicates that increased endogenous glucocorticoids may contribute to the bone fragility associated with old age. Thus, like patients treated with glucocorticoids, older individuals are ~10 times more likely to suffer fractures than younger individuals with the same mineral density (81). Similar to glucocorticoid excess, aging in mice is associated with increased osteocyte and osteoblast apoptosis and decreased bone strength that is not completely explained by a reduction in bone mineral density (82). Although Pyk2 null mice do not show gross anatomical alterations (83), recent studies revealed that these animals exhibit higher bone mass. This phenotype appears to result from defective osteoclast attachment and resorption leading to osteopetrosis (84) combined with increased bone formation and improved bone phores as well as growth factors (16, 39, 68, 69). A potential mechanism by which glucocorticoids could stimulate Ca²⁺ influx from the extracellular space is by increasing the production of reactive oxygen species (ROS) (70). In agreement with this contention, elevated levels of ROS activate Pyk2 in several cell types (71, 72).

Besides Pyk2, ROS elevation modulates the activity of members of the Rho family of small GTPases, focal adhesion-associated proteins involved in cytoskeleton organization and cell attachment (73). ROS up-regulates the activity of the small GTPase RhoA and the Rho-activated effector kinase ROCK (74). Activation of Rac1, another small GTPase, also depends on ROS production and leads to inhibition of endothelial cell adhesion (75). Moreover, RhoA, Rac1, as well as Cdc42 are all capable of activating the JNK pathway (73). Furthermore, JNK activation by Pyk2 depends on Rho/ROCK activation in vascular smooth muscle cells (76). Future studies are needed to determine whether anoikis of osteocytes induced by glucocorticoids involves changes in ROS production and/or the activity of small GTPases.

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